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Molecular Genetics of Forest Trees

Edited by W.M. Cheliak and A.C. Yapa

Petawawa National Forestry Institute
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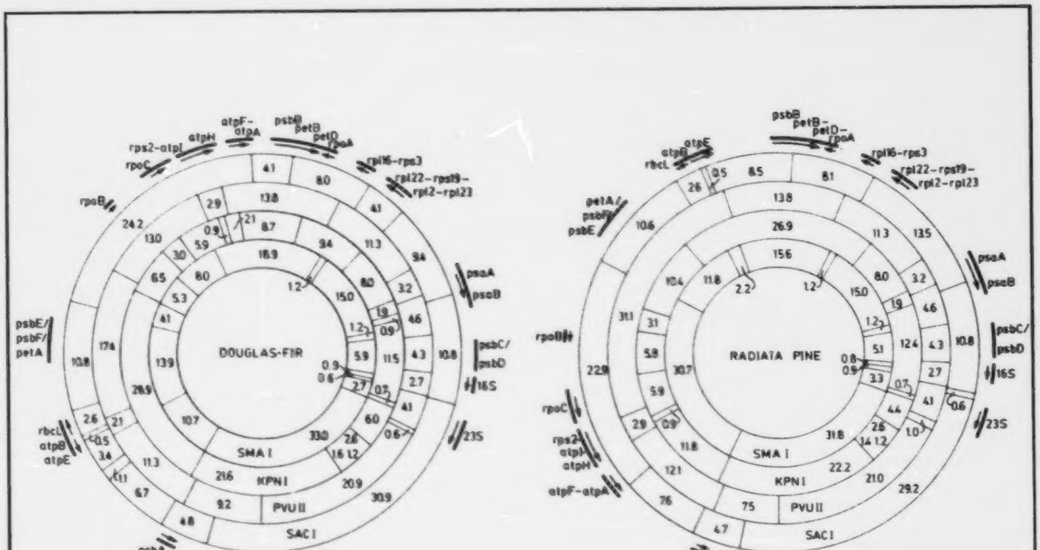
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RADIATA PINE
SMA I
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rpoA
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rpl22-rps19-rpl2-rpl23
rpoB
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psaA
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psbC/psbD
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PETAWAWA NATIONAL FORESTRY INSTITUTE

In common with the rest of the Canadian Forestry Service, the Petawawa National Forestry Institute has as its objective the promotion of better management and wiser use of Canada's forest resource to the economic and social benefit of all Canadians. Objectives of program activities carried out at the Institute support this goal through discovery, development, demonstration, implementation, and transfer of innovations. Because it is a national institute, particular emphasis is placed on problems that transcend regional boundaries or that require special expertise and equipment that cannot be duplicated in CFS regional establishments. Such research is often performed in close cooperation with staff of the regional centres, provincial forest services, and the forest industry.

Research initiatives and technical services at the Institute encompass six major activities:

FOREST GENETICS AND BIOTECHNOLOGY — Integrates projects in tree genetics, soil microbiology, micropropagation, molecular genetics, meteorology, and seed research. It also includes the client services and seed bank operations of the National Tree Seed Centre, a long-standing program with extensive international affiliations.

FOREST MANAGEMENT SYSTEMS — This program integrates projects in fire, remote sensing, modelling, and growth and yield to provide research and development for the formulation and demonstration of forest management systems.

NATIONAL FOREST RESOURCE STATISTICS — Provides biological, technical, and socioeconomic information on Canada's forest-based resources. The program involves progressive development of databases, such as FIDSINFOBASE, and establishment of new databases and software in support of policy development in forestry. The Forest Inventory Program collates information on the forest resource at a national level, maintains the Canadian Forest Resources Data System, and prepares the national forest inventory.

COMMUNICATIONS — Integrates activities of the library, public awareness, information, and editing and publications projects. The Institute is visited by more than 20 000 people every year. There is a Visitor Centre for the public, self-guided tours, and an extensive education project. The national repository of all scientific and technical publications of the CFS and the principal CFS publications distribution centre are both located at PNFI.

THE RESEARCH FOREST — Besides natural stands manipulated in a variety of ways for silvicultural research, the 98 km^2 Petawawa Forest contains extensive areas of plantations dating back six decades. Research plantations are a source of growth and yield data derived from cultural experiments, and they are becoming valuable for pedigreed genetic materials for micropropagation and molecular genetics studies. The forest also offers opportunities for short- and long-term testing of forest management strategies.

MOLECULAR GENETICS OF FOREST TREES

Edited by

W.M. Cheliak and A.C. Yapa

Proceedings, Second IUFRO Working Party on Molecular Genetics

Petawawa National Forestry Institute
Chalk River, Ontario, Canada
June 15-18, 1987

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Cover: Restriction site and gene maps of Douglas-fir and radiata pine chloroplast genomes. (see page 57)

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INTERNATIONAL UNION OF FORESTRY RESEARCH

The International Union of Forestry Research Organizations (IUFRO) is one of the oldest research organizations in the world. It was founded in the period 1890-1892 out of the need for international collaboration, organization, and intensification of research in forestry. IUFRO is worldwide and includes 100 member countries.

AIMS: IUFRO's objective is to promote international cooperation in scientific studies embracing the whole field of research related to forestry, including forestry operations and forest products, by facilitating the exchange of ideas, methods, data, and results among individual research workers throughout the world. These aims are accomplished by the development and maintenance of contacts among member organizations and their scientists, by encouraging the establishment of common programs of research, and by cooperation in carrying out these programs. IUFRO cooperates regularly with other international and national organizations. Each year, members organize 60 to 80 meetings, workshops, and excursions. Dozens of reports and proceedings of meetings are published every year and information is exchanged in the IUFRO News quarterly.

STRUCTURE: The International Council (IC) is the authority responsible for regulating the affairs of the Union. In particular, the IC originates and establishes major policies relating the programs of IUFRO and elects the President, Vice President, and Executive Board of the Union. The Executive Board acts as the executive organ of the Union. IUFRO also has a permanent Secretariat. Its address is: IUFRO Secretariat, Schönbrunn, A-1131 Vienna, Austria.

RESEARCH: Research is the primary activity of the Union. Within IUFRO there are more than 200 research groups organized for information exchange among over 15 000 scientists around the world. Operations are carried out through the six Divisions of IUFRO and their research groups. The activity of research groups is based on the initiative of the groups themselves and on joint divisional and interdivisional activities. Subject Groups and Working Parties may be established as needed. The activities of research groups are worldwide, although there is often a need for the organization of a regional subgroup.

IUFRO SPECIAL PROGRAM FOR DEVELOPING COUNTRIES: Sixty-three developing countries are represented in IUFRO, which has recently started a Special Program for Developing Countries to identify urgent forestry research, training, and development needs in the Third World. A Special Coordinator was named to undertake these new tasks of IUFRO.

IUFRO

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SECOND WORKSHOP OF THE IUFRO WORKING PARTY ON MOLECULAR GENETICS

Petawawa National Forestry Institute, Petawawa, Ontario, Canada
16-18 June 1987

IUFRO's Working Party on Molecular Genetics was established at the 1981 Congress in Kyoto, Japan. Its numerical designation, S2.04.06, places it in Division 2 (Forest Plants and Forest Protection), Subject Group 04 (Genetics), and Working Party 06 (Molecular Genetics, now also including Cytogenetics).

The Working Party's first meeting was held at Avon Lake, Ohio in October, 1985, by which time sufficient interest in the molecular genetics of forest trees seemed to warrant holding an initial small-group conference. The 1985 meeting, which was generally considered highly successful, was in the nature of a workshop, with 30 attendees, most of whom were active research workers. No proceedings were published because of the early state of research in this field of work, new as it was to forestry.

The second meeting of the Working Party continued the format of a small-group workshop. It was held in Canada, both for geographic diversification and in recognition of the strong new efforts undertaken by Canadian scientists working in this field. Dr. William Cheliak was local organizer and Co-chair of the Workshop. Because of the rapid development of forestry-related molecular genetics since the First Workshop, we decided to publish a proceedings. We are grateful for the support of the Canadian government in this undertaking.

At the beginning of 1987, I assumed new IUFRO responsibilities. This made it desirable for us to elect new Working Party officers for the remainder of the term, which expires December 31, 1990. Results of the balloting were announced at the Second Workshop. Dr. Michael Greenwood was elected Chairperson. For geographic diversity, and in recognition of new European work in this field, especially in Sweden, we elected as Co-chair, Dr. Peter Gustafsson. The new officers will be responsible for organizing the next meeting and any other activities of the Working Party during the period.

We look forward to further productive exchange of research techniques and progress in this rapidly-developing field, and to the participation of research colleagues in other countries in the activities of our Working Party.

Howard B. Kriebel, Chairperson
(September 1981 to September 1987)
IUFRO Working Party on Molecular Genetics

PARTICIPANTS

Second IUFRO Working Party on Molecular Genetics
Petawawa National Forestry Institute
Chalk River, Ontario, Canada
June 15-18, 1987

[PHOTO: STEVEN HANDKE]

Bottom row l→r: Glenn Howe, Steve Strauss, Marie-France Michel, Jonas Lindholm, Chris Cullis, Howard Kriebel, Merlin Leong, Claire Kinlaw, Malcolm Campbell, Stokes Baker, Joëlle Bercetche, Wang Ju

Top row l→r: Folke Sitbon, Michael Greenwood, Scott Schlarbaum, Russ Feirer, Scott Merkle, Petter Gustafsson, John Marshall, Bruce Dancik, Linda DeVerno, Christine Aitchison, Janet Chow, Suzanne Marshall, Keith Hutchison, Jack Pitel, Bruce Faiers, Dave Wagner, Bill Cheliak

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June 15-18, 1987

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ORGANIZERS' MUSINGS...

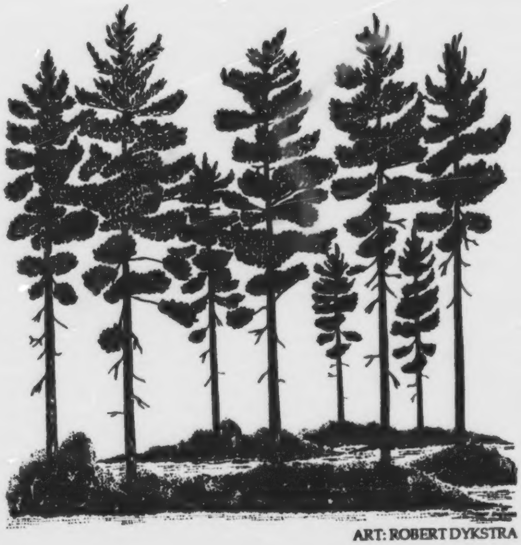
As Dr. Kriebel stated in his introductory remarks, there have been some rapid developments in the molecular genetics of forest trees during the two years since we held our first meeting at Avon Lake in Ohio. Given the small number of researchers, the significant technical difficulties working with trees, and the realities of having to patiently build and equip labs, it is fair to say that the accomplishments in the last two years have been nothing short of spectacular. Even the most optimistic scenarios two years ago could not have predicted this. It is evident that our persistence, dedication, and tenacity is paying off. Even from the teasing few glimpses we have of some aspects of forest tree molecular biology, it is clear that they provide a fascinating store of evolutionary history, developmental biology, and genome organization. For example, we have recently learned that the rDNA repeat in *Pinus radiata* is about 27 kb. This is about twice as long as that of any angiosperms studied to date. Unlike most other species studied so far, gymnosperms have a paternally, not maternally, transmitted chloroplast genome. Also during chloroplast evolution, in *Pinus contorta* and *P. banksiana*, the *psb*A gene is duplicated, but these species appear to lack one of the rRNA encoding repeats. Without a doubt, these early results are only the beginning of an exciting and fascinating time for those of us fortunate enough to be pioneers in the field of molecular biology of forest trees.

Every meeting has its special memories for the organizing committee. This one was no exception. Because we are about a 2 h drive from Ottawa, we had made specific arrangements for transportation of the conference participants from Ottawa to the Institute. Folke Sitbon, from the University of Umeå, who was not expected at the meeting, likely had the most interesting time getting to PNFI.

Due to flight delays and rerouting, Folke arrived very late in Ottawa and ended up having to take a bus from the city to the Institute. Folke got off of the bus at about 2:30 in the morning and decided that he would walk, with his luggage of course, to the main office from the highway. Those of you who know the Institute will appreciate the statement that it is located "in the forest". What Folke did not know was that this little walk would be about 3 km. Admittedly this was through some beautiful forest, but who can tell the difference in the middle of the night? What Folke was also blissfully unaware was that black bears and moose sometimes haunt this stretch of road. No doubt any bears that were around made a quick exit when they saw this heroic figure.

When Folke arrived at the main office some time later, no one was there to greet him. So he took up a comfortable position under a beautiful *Larix decidua* x *leptolepis* tree, presumably to look over his notes for the meeting. Several hours later one of our caretakers found Folke stirring to early signs of light and life around him. Soon our hero was enjoying a hearty breakfast in the cafeteria. Fortunately, the remainder of the meeting was not quite as exciting for Folke. We can assure you that, generally, we do not welcome our guests in quite the same fashion, and we laud Folke for his fortitude.

We wish to thank Eliza Andersen who keyed the text into the desktop publishing system at PNFI. Her extraordinary patience was tested time and again but she always came through, despite the fact that the system was new to her. Eliza's rapid and accurate typing made the editors' job so much easier.



ART: ROBERT DYKSTRA

CLONING cDNA OF CONIFER SEEDS

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Abstract

We describe the construction of a cDNA library of poly(A)$^+$ RNA from jack pine seed material. The poly(A)$^+$ RNA was obtained from both embryo and gametophyte tissues of seeds at several stages of imbibition and germination. Over 50% of the cDNA was of higher molecular weight than 500 bp, as determined by electrophoresis and autoradiography following labelling of the newly synthesized cDNA with [α-^{32}P]dCTP. Using the λgt10 viral based vector, up to 2.9×10^6 clear plaques (putative recombinants) per µg cDNA were obtained. Depending on the ligation mixture, up to 90% of clear plaques contained inserts. The ratio of L87/NM514 varied from 9.3 to 31.6, with highest ratios being obtained for cDNA that was added from 50-300 ng/µg of λgt10 arms. Jack pine inserts were subcloned into a pUC vector that was capable of transcribing the cDNA into RNA. This can be used for *in vitro* translation studies and also as a probe following labelling with ^{32}P.

Introduction

In recent years the cloning of complementary DNA(cDNA) copies of mRNAs has proven to be important in various plant molecular biology studies. One advantage of working with a cDNA library is that repetitive DNA in the complex plant genome is largely eliminated, facilitating analysis, whereas a library of genomic clones could be large and difficult to screen for individual genes. A cDNA library represents the transcriptional activity at a particular developmental and physiological state and thus contains only those gene segments that have been transcribed in that system, eg., leaves, seed embryos and gametophytes, roots, etc. Some applications of the cDNA cloning technique include: determining the number of loci corresponding to each gene (Bernatzky and Tanksley 1986); identifying the protein product of each cDNA clone, eg., lipoxygenase (Casey et al. 1985), in conjunction with other techniques such as hybrid-release translation and immunoprecipitation; analyzing the size and accumulation of specific mRNAs at different times and in different parts of the seed during germination and early seedling growth (eg., Galau et al. 1986, Datta et al. 1987); characterizing the cDNA's coding for auxin-regulated genes (Theologis et al. 1985, Kuhlemeier et al. 1987); identifying the molecular basis of disease resistance in plants (eg., Fristensky et al. 1985); and, characterizing the alteration of gene expression of plants due to environmental stress (eg., Sachs and Ho 1986).

We were interested in using the cDNA cloning technique to understand the mechanisms associated with imbibition and germination of conifer seeds. In a couple of recent reports with tamarack (*Larix laricina* [Du Roi] K. Koch) (Pitel and Cheliak 1986) and jack pine (*Pinus banksiana* Lamb.) (Pitel and Cheliak 1988), the activities of a number of enzymes was monitored quantitatively with a spectrophotometer during germination. For the embryos, enzyme activities were generally low in the dry seed and during imbibition but increased rapidly following emergence of the radicle and later germination.

In this report we describe the construction and preliminary characterization of a cDNA library of poly(A)$^+$ RNA from jack pine seed material. Using this library, and by applying other techniques, we hope to more clearly understand some of the control mechanisms regulating the expression of particular genes in tissues at different stages of imbibition and germination of conifer seeds.

Materials and methods

Seed material

Seeds of jack pine (*Pinus banksiana* Lamb.) were obtained from a single source at C.F.B. Petawawa, Ontario (lat. 45°59′, long. 77°23′W, elev. 150 m). Seeds were collected in 1981 and kept at -18°C until used for the experiment in early spring 1987. Germination was over 98%. Seeds were placed on sterile petri dishes and imbibed with sterile distilled water at 20°C with 8 h light and 16 h darkness. Dry seeds, one-day-imbibed seeds, three-day-imbibed seeds (just prior to radicle emergence), and seeds with radicles emerged were used for extraction. Seedlings of 8-12 mm and 15-20 mm in length were also utilized. At each stage of imbibition and germination embryo tissue was extracted from gametophyte tissue. Seed coats were discarded.

Chemicals and enzymes

Components for the cDNA synthesis system, the λgt10 cloning system, as well as *Eco*RI and *Hind*III were obtained from Amersham (Canada) Ltd. Ampicillin (sodium salt), ATP (disodium salt), Trizma acetate, Triton X-100, DL-dithiothreitol, diethyl pyrocarbonate, MOPS, IPTG (isopropyl-β-D-galacto pyranoside, X-gal (5-bromo-4-chloro-3-indole-β-D galacto-pyranoside), Sigmacell (type 50), sodium phosphate (dibasic), ethidium bromide, 2-mercaptoethanol, Proteinase K, rubidium chloride, DNA (Salmon sperm, Type III), EGTA, potassium acetate, bromcresol green, and polyethylene glycol 8000 were all purchased from Sigma Chemical Co. Lithium chloride was obtained from Anachemia Chemicals Ltd. Phenol (ultra pure), Tris (ultra pure), and cloned M-MLV reverse transcriptase were purchased from BRL. Bacto-yeast extract, Bactotyptone, and Bacto-agar were obtained from Difco Laboratories. Disodium EDTA, NaOH, sodium borate, sodium acetate, magnesium sulfate, and sodium lauryl sulfate were from Fisher Scientific. DEAE-cellulose (Whatman) DE52 (preswollen, microgranular) was obtained from Mandel Scientific. Trichloroacetic acid, NaCl, and ammonium acetate were from BDH. Ficoll (type 400) and Agarose NA were obtained from Pharmacia. Calcium chloride was purchased from J.T. Baker Chemical Co.

Extraction of total RNA

Dissected seed material was stored at -80°C until extraction, which usually occurred within a couple of weeks following dissection. Depending on the stage of imbibition and germination 5-20 g of seed material was ground to a fine powder in liquid N_2 and extracted essentially as described by H. Kreibel (pers. comm.). The usual precautions were taken to prevent the action of ribonucleases, such as heat-sterilizing glassware, autoclaving where appropriate, and the use of diethyl pyrocarbonate. Following addition of the heated extraction buffer (0.2 M sodium borate pH 9.0, 30 mM EDTA, 1% SD; 5 mM dithiothreitol) the mixture was ground for 1 min with a Polytron or Waring blender. The mixture was filtered through one layer of Miracloth, and self-digested Proteinase K added (for 10 g of tissue, 13 mg of Proteinase K was digested with 2 mL of Tris-acetate buffer, pH 7.5). The mixture was incubated at 37°C for 1 h and 2 mL of 2M KCl (for 25 mL of extract) added. Following centrifugation at 10 000 rpm for 10 min at 4°C, 1/4 volume of 10M LiCl was added. The mixture was placed at -70°C for 20 min and then at 4°C for 16 h. After centrifugation at 4°C for 10 min at 10 000 rpm, the pellet was washed with 2M LiCl and then suspended in a few mL of 2M LiCl using a suitable tissue grinder. The mixture was centrifuged at 10 000 rpm for 10 min and the pellet resuspended 2-3 times additionally. The final pellet was washed with 2M LiCl and then dissolved in a few mL of sterile distilled water. To this was added 50 µl/mL of 3M sodium acetate and 2.5 volumes of cold 95% ethanol, and the solution then placed at -20°C for 16 h. The RNA-containing precipitate was pelleted by centrifugation at 10 000 rpm for 10 min and dissolved in 2 mL of phenol extraction buffer (10mM Tris-HCl, pH 9.0, lmM EDTA, 0.1% Triton X-100). The solution was extracted with buffer-saturated phenol and, after centrifugation, the top phase was saved while the bottom phenol phase was extracted with an equal volume of phenol extraction buffer. The combined aqueous phases were then extracted several times with an equal volume of phenol/chloroform/isoamyl alcohol (24:24:1). To the final aqueous phase was added 50 µl/mL of 3M sodium acetate and 2.5 volumes of cold 95% alcohol. The precipitate was centrifuged at 10 000 rpm for 10 min, the pellet dissolved in 2 mL of elution buffer (10mM Tris-HCl, pH 7.6, 1mM EDTA, 0.1% SDS), and then 100 µL of 3M sodium acetate and 5 mL of cold 95% ethanol was added. The mixture was kept at -20°C for 16 h and afterwards the RNA was passed through a Sigmacell Type 50 cellulose column.

Oligo (dT) cellulose chromatography

Poly(A)$^+$ RNA was separated from rRNA by oligo (dT) cellulose (BRL) chromatography essentially as described by Maniatis et al. (1982) and Perbal (1984). The RNA was stored at -80°C in 70% ethanol. Analysis of poly(A)$^+$ RNA was by electrophoresis using formaldehyde gels (Perbal 1984).

Preparation of cDNA against poly(A)$^+$RNA

The first strand reaction mix included 1-5 µg poly(A)$^+$RNA, 20-40 units of reverse transcriptase, oligo-dT$_{12\text{-}18}$ as a primer, deoxynucleoside triphosphate mix, human placental ribonuclease inhibitor, sodium pyrophosphate solution, suitable buffer (as obtained from Amersham (Canada) Ltd.), and 5 µCi of [α-^{32}P] dCTP per 20 µL of reaction. The solution was incubated at 42°C for 2 h. To this reaction mixture, on ice, was added a suitable buffer, 50 µCi of [α-^{32}P] dCTP, 0.8 units of *E. coli* ribonuclease H, 23 units of *E. coli* DNA polymerase I, and water to 100 µL. After incubation at 12°C for 60 min, 22°C for 60 min, and 70°C for 10 min, 2-4 units of T4 DNA polymerase was added and the mixture incubated at 37°C for 10 min. The reaction was stopped by adding 10 µL of 0.25M EDTA pH 8 and 10 µL of 10% SDS. Double-stranded cDNA was purified by phenol/chloroform and ethanol precipitation. The products of the cDNA synthesis reactions were analyzed by electrophoresis on a 1.4% agarose gel under denaturing conditions (50 mM NaOH). Following electrophoresis, the gel was soaked for 30 min at room temperature in 7% trichloroacetic acid (two changes). The gel was dried, covered with plastic wrap, and the cDNA detected by autoradiography at -70°C for 2-4 days using Kodak X-Omat AR film and intensifying screens. Before loading on the gel, each sample represented about 500-1000 cpm of radioactivity.

Cloning

Cloning of cDNA fragments was done according to specifications of the λgt10 cloning system of Amersham. For ligation, various amounts (50-800 ng) of insert DNA were added to the reaction. In addition to the plating cells obtained from Amersham (*E. coli* L87 and *E. coli* NM514(hfl$^+$)), *E. coli* C600 and *E. coli* C600(hfl$^+$) (Promega Biotec) were also tested.

Characterization of inserts

To detect the presence and size of an insert, DNA was purified and then digested with either *Hind*III or *Eco*RI for 1-2 h. Electrophoresis was with 0.8% agarose (for *Hind*III digests) or 1.8% agarose (for *Eco*RI digests).

Filter hybridization

Following plating of phage, replica filters were obtained using Hybond-N 82 mm circles (Amersham). The denaturation solution was 0.5M NaOH, 1.5M NaCl. Following transfer to a neutralizing solution (0.5M Tris-HCl pH 7.0, 1.5M NaCl) for 5 min, rinsing in 2XSSC, and baking at 80°C for 2 h, the filters were hybridized with the first strand cDNA synthesis mixture (obtained from embryo poly(A)$^+$ RNA at the radicle emerging stage) at 65°C for 16 h. Following washing of the filters (as described by Amersham) they were then autoradiographed at -70°C with intensifying screens to identify positive plaques.

Transcription system

Following phage DNA purification of the clear plaques, the DNA was digested with *Eco*RI for 1-2 h at 37°C and then analyzed by agarose electrophoresis (1.8% gels). Single jack pine cDNA bands with sizes of 500 bp or greater were cut out from the gel and the DNA electroeluted (International Biotechnologies, Inc.). Jack pine cDNAs of different sizes were then ligated into a pGEM-3Z plasmid DNA vector (Promega Biotec). Transformation was with a restriction minus strain (JM 109). The above vector has the SP6 and T7 promoters for transcription, as well as providing blue/white selection for recombinant clones. Plasmid DNA was extracted from white colonies using either the method of Birnboim and Doly (1979) or by the "Geneclean" system (BI0 101, La Jolla, CA.).

Results

Extraction of poly(A)$^+$RNA

For the RNA samples extracted to date, the percentage of poly(A)$^+$RNA to that of total RNA ranged from 0.9-1.6%. The yield of poly(A)$^+$RNA on a fresh weight basis varied with the period of imbibition and germination, and also with the type of tissue. The highest yield was obtained from embryos following radicle emergence, with a content of 41 µg poly(A)$^+$RNA per g fresh weight. Yields of poly(A)$^+$RNA from gametophyte tissues were lower than for embryos. There was no evidence of any contaminating material in the poly(A)$^+$RNA samples that could be detected at 280 nm. Electrophoresis of the poly(A)$^+$RNA resulted in a smear from a region of 5S RNA to well over 25S RNA. Most of the staining intensity occurred in a region of 14-16S RNA. There was some evidence for contamination by rRNA (< 5%). However, no band of 18S rRNA was observed.

Preparation of cDNA

As shown in Figure 1 (lane 1) only a single band was observed for cDNA produced from 10 S globin mRNA. However, for the cDNA obtained from jack pine poly(A)$^+$RNA a heterogeneous pattern was obtained that contained cDNA from about 200 bp to over 2000 bp. Most of the cDNA was present in a region of just over 560 bp. However, for one extract (lane 6) in which poly(A)$^+$RNA was added to the cDNA reaction mixture in excess, the molecular weights of the cDNA's shifted to a lower molecular weight region. The cDNA from lane 7 represents only single-stranded cDNA.

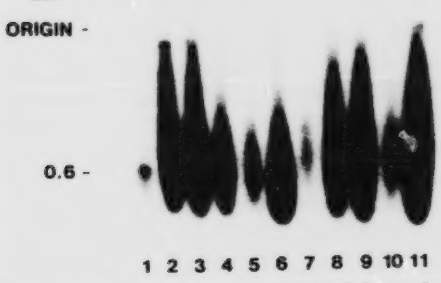

Figure 1. An analysis, by agarose (1.4%) gel electrophoresis, of the product size distribution of jack pine cDNA from embryos at two stages of germination. Lane 1: cDNA from 10 S rabbit globin mRNA; Lanes 2-6: cDNA from mRNA of embryos at radicle stage of emergence (mRNA added was 1, 2, 3, 3, and 5 µg for lanes 2-6, respectively); Lanes 7-11: cDNA from mRNA of seedlings with 10-15 mm in length (mRNA added was 1, 2, 2, 2, and 3 µg for lanes 7-11, respectively).

Cloning

As shown in Table 1, different numbers of clear plaques (putative recombinants) were obtained when different amounts of cDNA were added to the λgt10 ligation reaction. Highest numbers (per µg cDNA) were obtained when 300 ng of cDNA was used per µg of λgt10 arms. The L87:NM514 ratios were highest for decreasing amounts of cDNA added to the ligation mixture. Different results were observed when using *E. coli Hfl*$^+$ strains NM514 and C600. For the latter, plaque sizes were more variable, there was more contrast between clear and opaque plaques, and the number of plaques was slightly greater than for the NM514 strain.

Depending on the ligation mixture, the number of clear plaques that contained inserts varied between 50-90%. Figure 2 shows the sizes of some of the jack pine cDNA inserts. Digestions

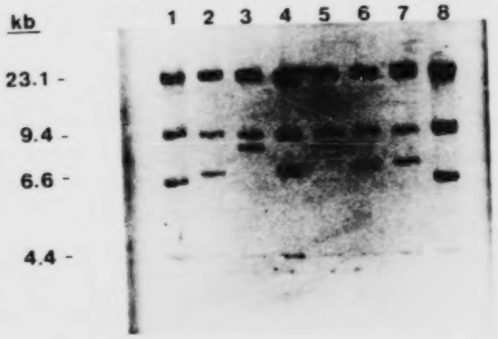

Figure 2. An analysis, by agarose (0.8%) gel electrophoresis, of DNA purified from clear plaques and digested with *Hind*III. Lanes 1,8: Lambda DNA-*Hind*III digest control showing base pair sizes of 23 130, 9416, 6557, and 4361 starting from the top. Lanes 2-7: plaques with inserts are shown having bands with a size different and greater than that of the control size of 6557 bp.

Table 1. Results of cDNA cloning experiments reporting the number of putative recombinants per µg jack pine cDNA (obtained from poly(A)$^+$ RNA of embryos at the radicle stage).

Insert (cDNA)	L87 titre	NM514 titre (clear plaques)	L87:NM514 ratio	Recombinants per µg cDNA
50 NG	1.58×10^6	0.05×10^6	31.6	1.00×10^6
200 NG	3.90×10^6	0.19×10^6	20.5	0.95×10^6
300 NG	22.62×10^6	0.87×10^6	26.0	2.90×10^6
500 NG	7.20×10^6	0.65×10^6	11.1	1.30×10^6
800 NG	1.70×10^6	0.18×10^6	9.3	0.23×10^6

were also done using *Eco*RI, however, more material was required on the gel for the insert bands to be clearly visible.

Filter hybridization

The use of the radioactive first strand synthesis mixture as a probe resulted in signals being obtained on the sites of plaques. However, the intensity of the signal varied considerably with the plaque; some were very intense while others were weak or even lacking any signal. The same probe was also capable of hybridizing to poly(A)$^+$ RNA of embryos at two stages of germination following a Northern blot. No obvious differences in pattern were observed between the two stages of germination (radicle stage, and two days later, germination).

Transcription system

Jack pine cDNA inserts that were electroeluted from the gels, as described in the Materials and Method section, were ligated to the pGEM-3Z plasmid DNA vector and then transformed into JM 109 cells. Following plating on LB plates that included 100 µg/mL ampicillin, 40 µg/mL X-Gal, and 0.5 mM IPTG, both blue and white colonies were observed. The use of alkaline phosphatase (calf intestine) was required to decrease the background of recircularized plasmid vector molecules. DNA extraction of plasmids from the white colonies resulted in the formation of two bands following electrophoresis. One band was the vector molecule migrating at 2.75 kb; the other lower molecular bands were the putative cDNA inserts. For many of the colonies the molecular weights of the cDNA inserts were similar to that of the original bands that were first cut from the gels and used for this ligation experiment. However, for some colonies, a band was obtained with an unexpected molecular weight. Both methods of extraction of plasmid DNA resulted in clear bands and, with the "Geneclean" method, RNA contamination was minimized.

Discussion

Our results have shown that high yield, physically intact poly(A)$^+$RNA can be isolated from imbibed and early germinating seeds of jack pine. However, we are trying other methods to increase the speed and effectiveness of extraction, such as the use of guanidinium thiocyanate as a protein denaturant and "Hybond" (Amersham) to replace time-consuming column purification of poly(A)$^+$RNA. Although we used the Amersham system for synthesis of cDNA from our poly(A)$^+$RNA, other systems are available. For example, the BRL cDNA synthesis system uses cloned Moloney murine leukemia virus (M-MLV) reverse transcriptase, which is reported by BRL to be a much purer source of the enzyme than other types such as avian myeloblastosis viral (AMV) reverse transcriptase. The large molecular weight range of our cDNA (Figure 1) (average size of about 550 bp) corresponds to a similar heterogeneous pattern for the poly(A)$^+$RNA, and the large size of some of the cDNA indicates that many full-length transcripts are probably present.

Our λgt10 cloning system resulted in the production of many recombinants and was a much more efficient and less complicated system than when we tried pBR322 as a cloning vector. Although most clear plaques contained inserts, the cDNA library should be tested for the frequency of rRNA sequences, especially if the poly(A)$^+$RNA is not sufficiently purified or if the rRNA is partially degraded, exposing internal A-rich regions.

Following digestion of the DNA obtained from the clear plaques, the jack pine cDNA inserts can be subcloned into a suitable vector such as pGEM-3Z plasmid DNA (Promega). We are in the process of using this vector to produce high specific activity RNA probes for Northern and Southern hybridization studies, as well as for *in vitro* translation and sequencing studies. We are also testing the "Lambda Zap" system (Stratagene, San Diego, CA.), which eliminates the need for subcloning; the cDNA's contained in the "Lambda Zap" are automatically excised into plasmid vectors. Various experiments can then be conducted including sequencing, synthesis of RNA probes, and expression of proteins.

One area we will be exploring, in which the above cDNA techniques will be useful, is in understanding some of the mechanisms associated with growth and differentiation during seed germination at the level of gene regulation. With the onset of imbibition, protein synthesis is rapidly activated and thus transcriptional, post-transcriptional, and translational regulations are in operation. Preformed mRNA is present in many dry seeds, including Japanese red pine *Pinus thunbergii* Parl. (Yamamoto 1982), and these may code for proteins playing a specific role in early germination (Aspart et al. 1984). However, further studies are needed in this area as there are many conflicting reports about the role of preformed mRNA, in early imbibition and germination. As the gametophyte tissue accumulates reserve material with seed maturation and then hydrolyzes them following germination as nutrients for the growing embryo, there should be marked changes in gene expression during this transition period.

Once cDNA libraries are constructed specific cDNA clones could be identified by differential hybridization of mRNA molecules present at one stage of development but absent in another. With the use of ^{32}P-labelled cDNA clones, the expression of mRNA homologous to the cDNA clones can be monitored with development and germination, as well as the expression in different tissues (eg., Mesnard et al. 1985). In a study with cotton (*Gossypium hirsutum* L.) cotyledons, Galau et al. (1986) obtained cDNA clones for 18 mRNAs which were more abundant during late embryogenesis than in mid-embryogenesis or early germination. Levels of each of these mRNA sequences were tenfold to several hundred-fold higher than young embryo or 24 h seedling poly(A)$^+$RNA. Polypeptides encoded by each mRNA sequence can be characterized by hybrid-arrested translation, or by subcloning of the cDNA into a plasmid vector that has promoters capable of transcription. The identity of some of the resulting polypeptides can be examined by immunological techniques (eg., Chrispeels et al. 1984). Western blot analysis can be used to examine polypeptide accumulation. If specific radioactive probes are available (eg., synthetic oligonucleotides, heterologous probes, protein-specific cDNA clones, antisera against individual proteins) library (eg., λgt11 or "Lambda Zap" for antibody screening) can be screened directly for identity of clones.

Certain aspects of gene expression control with developing cotyledons of *Phaseolus vulgaris* were examined by Chappel and Chrispeels (1986). They used nuclear runoff transcription assays, together with assays of the relative mRNA amounts measured in RNA blot hybridization of certain genes, to study the regulation of storage protein synthesis. They observed that the transcription rate of the phaseolin genes during cotyledon development increased rapidly and then subsequently declined. Because this decline was accompanied by a maintenance of mRNA levels, the authors suggested that mRNA abundance was regulated at the level of transcription and also by post-transcriptional events.

In a study with soybean (*Glycine max* (L.) Merr.) seeds Walling et al. (1986) showed that, although seed protein genes are transcriptionally induced and then repressed during embryogenesis, post-transcriptional processes (eg. cytoplasmic entry rates and/or differential mRNA stabilities) are also important in regulating seed protein gene expression. The authors observed, as well, that transcriptionally active and inactive seed protein genes have similar methylation patterns and that seed protein genes are under-methylated in relation to average soybean DNA regions. In a further step, gene transfer systems are needed to identify the DNA sequences and cellular factors that are involved in the regulation of seed protein gene expression.

The regulation of synthesis of certain proteins such as gliadin (alcohol-soluble wheat storage proteins), in addition to being controlled at the transcriptional level, can also be partly controlled by the activity of the protein synthesis machinery (eg., Tercé-Laforgue et al. 1987). Cloned cDNA probes were used by Greenland et al. (1987) to measure steady-state transcript levels in the cotyledons of developing cucumber (*Cucumis sativus* L.) seedlings for the small subunit (SSU) of ribulose bisphosphate carboxylase-oxygenase and the light-harvesting chlorophyll *a*/*b*-binding protein (LHCP). They showed that accumulation of transcripts to SSU occurred independent of light (although at reduced levels in the dark) while, in contrast, accumulation of transcripts to LHCP occurred only in light-grown cotyledons. They also showed that there was one gene encoding SSU and two genes encoding LHCP following Southern blot analysis of genomic DNA. In a study by Borroto and Dure (1986) several chloroplast genes (for ATPase, chloroplast rDNA, Photo Gene, RUBP carboxylase LSU) were expressed during cotton embryogenesis at substantial levels, even though the cotyledons were not exposed to light and are not green at any embryonic stage. Temporal as well as spatial changes in mRNA populations have been observed by Datta et al. (1987) during germination and early seedling growth of soybean (*Glycine max* L.) Merr.).

Summary

We have been able to make cDNA libraries of poly(A)$^+$ RNA during imbibition and germination. We are presently studying the isolation and characterization of some tissue-specific and stage-specific cDNA clones. Such investigations will provide insights into the mechanisms of gene regulation with growth and differentiation.

Acknowledgments

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USE OF HETEROLOGOUS PROBES TO ISOLATE AND CHARACTERIZE CONIFER GENES

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Abstract

The feasibility of using nucleic acid probes from an angiosperm (*Zea mays* L.) to isolate and characterize alcohol dehydrogenase and aldolase cDNAs from a gymnosperm (*Pinus radiata* D. Don) is demonstrated.

Introduction

Advances in understanding the molecular mechanisms which regulate expression of a number of plant genes, including alcohol dehydrogenase (*Adh*), have been due primarily to the ability of researchers to: 1) isolate *Adh* from several species, including *Zea mays* L. (Dennis et al. 1984, 1985) and *Arabidopsis thaliana* (Chang and Meyerowitz 1986); 2) determine plant gene DNA sequences; 3) modify specific sequences within these *Adh* genes; and 4) reintroduce these modified sequences into plant cells to study their expression (Fromm et al. 1985, Ellis et al. 1987, Howard et al. 1987). These advances have been limited almost entirely to angiosperms, and little is known about the mechanisms which regulate gene expression in gymnosperms. The application of molecular genetic techniques promises similar advances for the biologically and economically important gymnosperm group. Comparison of DNA sequences for the structural and regulatory portions of genes from angiosperms versus gymnosperms will allow refinement of estimates of genetic distances between these taxa, and will provide insights into the evolution of regulatory mechanisms.

The *Adh* genes are of interest because they display several levels of regulation (for reviews see Bennett and Freeling 1987, Freeling and Bennett 1985). In a number of angiosperms, including tomato (Tanksley and Jones 1981), barley *Hordeum vulgare* (Hanson et al. 1984), and maize (reviewed in Freeling and Bennett 1985) *Adh* genes are regulated spatially and temporally during development. In addition to this developmental regulation, *ADH* proteins are members of a set of specific proteins which are induced in response to anaerobic stress (Hageman and Flesher 1960, Sachs and Freeling 1978, Sachs et al. 1980, Hanson et al. 1984, Mayne and Lea 1984, Dolferus et al. 1985, Ricard et al. 1986, Van Geyt and Jacobs 1986).

There is evidence that *Adh* genes in conifers are also regulated during development (Barnett and Naylor 1969, Conkle 1971) and during anaerobic stress (DeBell et al. 1984, and Harry et al. 1987). Thus, these genes are logical candidates for a detailed study of the molecular mechanisms responsible for complex regulatory patterns in gymnosperms.

As an aid to studying *Adh* regulation in gymnosperms, we demonstrate here that nucleic acid probes from an evolutionary divergent organism, corn (*Zea mays*), will hybridize to Monterey pine (*Pinus radiata*) sequences under conditions of low stringency, and that these heterologous probes can be used to detect and isolate cDNA sequences from a Monterey pine cDNA library.

Materials

We obtained standard chemical reagents from Sigma Chemical Co. The enzymes used, except those listed in Table 1, were from New England Biolabs. For other biological reagents please see Table 1.

Table 1. Sources of materials

MATERIAL	SOURCE
Enzymes	
Avian myeloblastosis virus reverse transcriptase	Seikagaku America, Inc.
High concentration *Eco*RI	Boehringer Mannheim Biochemicals
Nuclease SI	Boehringer Mannheim Biochemicals
DNA Sequenase kit	US Biochemicals
Proteinase K	Sigma Chemical Co. or Bethesda Research Laboratories
lambda *in vitro* packaging extracts	Clontech
[32]P-deoxynucleotide triphosphates	New England Nuclear
[35]S-deoxynucleotide triphosphates	New England Nuclear
*Eco*RI linkers 5′-hydroxyl-d(GGAATTCC)	Promega Biotech
Oligo-dT-cellulose	Collaborative Research, Inc.
Hybond-N and Hybond-C membranes	Amersham
NA45 membrance	Schleicher and Schuell
Phenol (ultra pure)	Bethesda Research Laboratories
Chloroform (AR grade)	Baker Chemical Co.
Bacterial media components	Difco
Bacterial strains and phage strains	
C600 hfl	Clontech
λgt10	Clontech
M13mp19	Promega Biotech
JM107	gift from J. Messing
DNA probes	
Maize cDNA clone pZML793 (Adh1F)	gift from E. Dennis
Maize cDNA clone pZML154 (Ald)	gift from P. Kelly
Maize RNA	
Poly(A)$^+$ RNA from anaerobically-induced maize seedlings	gift from B. Kloeckner and M. Freeling
Computer software	
DNA Inspector II for Apple Macintosh	TEXTCO

Methods

Nucleic Acid Isolation

Monterey pine suspension line: A green and rapidly dividing suspension line of Monterey pine was maintained in P6 medium and transferred every 7-10 days. (R. Teasdale, pers. comm.)

Genomic DNA isolation: High molecular weight DNA was isolated from Monterey suspension cells using a modification (Stomp et al. 1987) of the procedure of Zimmer et al. (1981).

RNA isolation: Total cellular RNA was isolated according to the procedure of Lizardi and Engleberg (1979) and poly(A)$^+$ RNA was fractionated on oligo dT-cellulose according to the manufacturer's (Collaborative Research) recommendation.

Hybridizations

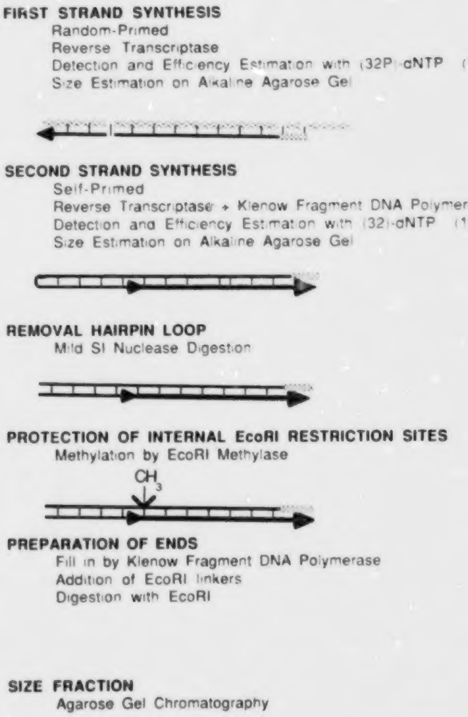
Radiolabelled probes: cDNA inserts were gel purified from either pZML793 (*Adh*1, Dennis et al. 1984) or pZM1154, aldolase (*Ald*, Kelley & Tolen 1986), using NA45 paper according to the manufacturer's (Schleicher and Schuell) suggestions except that the DNA fragments were eluted from the NA45 paper in 10 mM TRIS, pH 8.0, 1 mM EDTA, and 1M NaCl. The DNA fragments were radiolabelled using the random hexamer method (Barta et al. 1981).

Genomic Southern Blot: DNA was digested with restriction enzymes, fractionated electrophoretically through a 0.8% agarose gel, and transferred to a nitrocellulose membrane in 15X SSC (Maniatis et al. 1982) Hybridization of genomic DNA to the maize *Ald* probe was performed in 6X SSC at 65°C. (Maniatis et al. 1982) and post-hybridization washes were, first, 4X SSC at 55°C and, second, following autoradiography of the blot, by 0.5X SSC at 65°C.

Northern Blot: Total RNA was fractionated electrophoretically through denaturing agarose gels containing formaldehyde (Lehrach et al. 1977) and transferred to a nylon membrane, Hybond-N. Hybridization to the maize *Adh*1 cDNA clone was performed in 6X SSC at 55°C and the blot was washed under low stringency conditions, 1X SSC at 45°C (Maniatis et al. 1982).

cDNA Isolation

cDNA Synthesis: RNA was selected three times on oligo-dT-cellulose and double-stranded cDNA was synthesized using the procedures of Huynh et al. (1985) as outlined in Figure 1. Random oligonucleotides were used to prime the first strand synthesis in place of oligo-dT. Following cDNA synthesis, repair of DNA ends, methylation to protect internal *Eco*RI sites, attachment of *Eco*RI linkers, and digestion with *Eco*RI, the cDNAs were analyzed for size by alkaline gel electrophoresis (Maniatis et al. 1982) after first strand synthesis, second strand synthesis, SI nuclease digestion, and size fractionation.


FIRST STRAND SYNTHESIS
Random-Primed
Reverse Transcriptase
Detection and Efficiency Estimation with (32P)-dNTP (1X)
Size Estimation on Alkaline Agarose Gel
SECOND STRAND SYNTHESIS
Self-Primed
Reverse Transcriptase + Klenow Fragment DNA Polymerase
Detection and Efficiency Estimation with (32)-dNTP (10X)
Size Estimation on Alkaline Agarose Gel
REMOVAL HAIRPIN LOOP
Mild SI Nuclease Digestion
PROTECTION OF INTERNAL EcoRI RESTRICTION SITES
Methylation by EcoRI Methylase
CH3
PREPARATION OF ENDS
Fill in by Klenow Fragment DNA Polymerase
Addition of EcoRI linkers
Digestion with EcoRI
SIZE FRACTION
Agarose Gel Chromatography

cDNA Library Construction and Screening: cDNAs larger than 500 bp were pooled and ligated into dephosphorylated λgt10 arms and packaged *in vitro* according to manufacturer's (Clontech) instructions. An aliquot of the phage library was plated onto the host strain C600 hfl in order to determine the titer of the library. The entire library was then plated onto 150 mm petri dishes at a density of 5 x 10^4 plaque forming units (pfu) per dish.

Two pairs of duplicate plaque lifts were prepared from each petri dish on either nitrocellulose membrane (Hybond-C) or Hybond-N. The lifts were prepared according to the methods of Maniatis et al. (1982), and the phage DNA on a pair of filters from each petri dish was hybridized to either the *Adh*1 or the *Ald* cDNA probe under conditions established for Southern and Northern blot hybridizations (hybridization in 6X SSC at 55°C, and washes in 4X SSC at 55°C).

Individual plaques showing positive hybridization on both duplicate filters were amplified and plaque-purified by another round of plating on C600 bacterial lawns. Each clone was then hybridized again to either *Adh*1 or *Ald*. Clones which showed positive hybridization through these two rounds of screening were selected for further analysis.

cDNA Clone Characterization

DNA Isolation from Individual *Adh* Clones: Phage from positive *Adh* clones were plated at 5 x 10^6 pfu per dish onto 10-20 150 mm petri dishes containing LB agarose medium supplemented with 0.2% glucose and grown for 5-6 hours until the bacterial lawns developed a mottled appearance (Huynh et al. 1985). Each petri dish was overlayed with 10 mL of 10 mM Tris HCl pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, and 100 mM NaCl (TMEN), along with a few drops of chloroform and held at 4°C overnight. The bacterial debri in the overlay was collected by centrifugation at 10 000 x g, 4°C, 15 min. The titer of each phage stock was estimated by gel electrophoresis of an aliquot according to the procedure of Xu (1986) or determined directly. Only stocks of high titer (approximately 10^{10} pfu/mL) were used for DNA isolations.

The phage was precipitated by addition of NaCl to 1M and polyethylene glycol 8000 to 10% at 4°C and collected by centrifugation at 10 000 x g for 15 min. at 4°C. (Maniatis et al. 1982). Contaminating bacterial RNA and DNA were removed by a modification of the DEAE-adsorption procedure of Xu (1986). The phage were resuspended in TMEN and an equal volume of a DEAE-cellulose slurry in TMEN was added. The phage were mixed by inversion for 3 min at room temperature and then DEAE-cellulose was removed by centrifugation in a clinical centrifuge. The phage was precipitated by the addition of 0.125 mL, 5 M NaCl, and 0.78 mL isopropanol per mL stock, incubation for 30 min at -20°C, and centrifugation at 10 000 x g for 15 min at 4_oC.

A small aliquot of phage was again analyzed by electrophoresis through an agarose gel to determine the extent of residual contamination by bacterial RNA and DNA. If required, RNAse (10 μg/mL) and DNAse (1 μg/mL) were added and the phage incubated for 20 min at 37°C to digest any remaining bacterial nucleic acids. The phage were lysed by proteinase K digestion (50 μg/mL) for 20 min in the presence of 0.2% SDS at 37°C followed by extraction with buffered phenol:chloroform (Maniatis et al. 1982). Residual phenol was removed by extraction with chloroform, followed by extraction with water-saturated ether. Phage DNA was precipitated by the addition of 0.1 volume of 3M sodium acetate and two volumes of ethanol, and collected by centrifugation at 12 000 x g for 10 min at 4°C.

Clone Characterization: DNA from individual lambda clones was digested with restriction enzymes, fractionated electrophoretically through 1% agarose gels, blotted to Hybond-N nylon membrane, and hybridized to the maize *Adh*1 probe under conditions used for screening the library. Pine cDNA inserts were gel purified following digestion of lambda clones with *Eco*RI and subcloned into *Eco*RI-digested M13mp19 (Messing 1983). Single-stranded DNA templates were sequenced by the chain termination method of Sanger et al. (1979), using T7 DNA polymerase as described by Tabor and Richardson (1985) under conditions recommended by the Sequence kit supplier (US Biochemicals). DNA sequences of pine *Adh* clones were compared to the maize *Adh*1F DNA sequence using the DNA sequence analysis software package, DNA Inspector II, on an Apple Macintosh computer (Gross 1986).

Results and Discussion

Hybridization of Pine Sequences to Heterologous Probes at Reduced Stringencies

To test the feasibility of using heterologous probes for isolating *Ald* and *Adh* cDNAs and genomic DNAs from pines, we performed hybridization experiments under reduced stringencies. The maize *Ald* probe hybridizes to Monterey pine genomic DNA under low stringency conditions, 4X SSC and 55°C, but not under high stringency conditions of 0.5X SSC (Figure 2, part A). Assuming a 40% GC content for the *Ald* sequences, these hybridization data suggest that the *Ald* gene(s) from Monterey pine shares approximately 70% homology with the maize *Ald* coding sequence.

Under the same low stringency conditions (4X SSC and 55°C) which allowed hybridization of maize *Ald* to Monterey pine genomic DNA, the maize *Adh*1 probe failed to hybridize. However, the maize *Adh*1 probe did hybridize under similar low stringency conditions (1X SSC and 45°C) to Monterey pine poly(A)$^+$ RNA (Figure 2, part B). These hybridization data suggest that *Adh* coding sequences are more highly conserved between maize and Monterey pine than *Adh* noncoding sequences, and that *Ald* genomic sequences are more highly conserved than *Adh* genomic sequences.

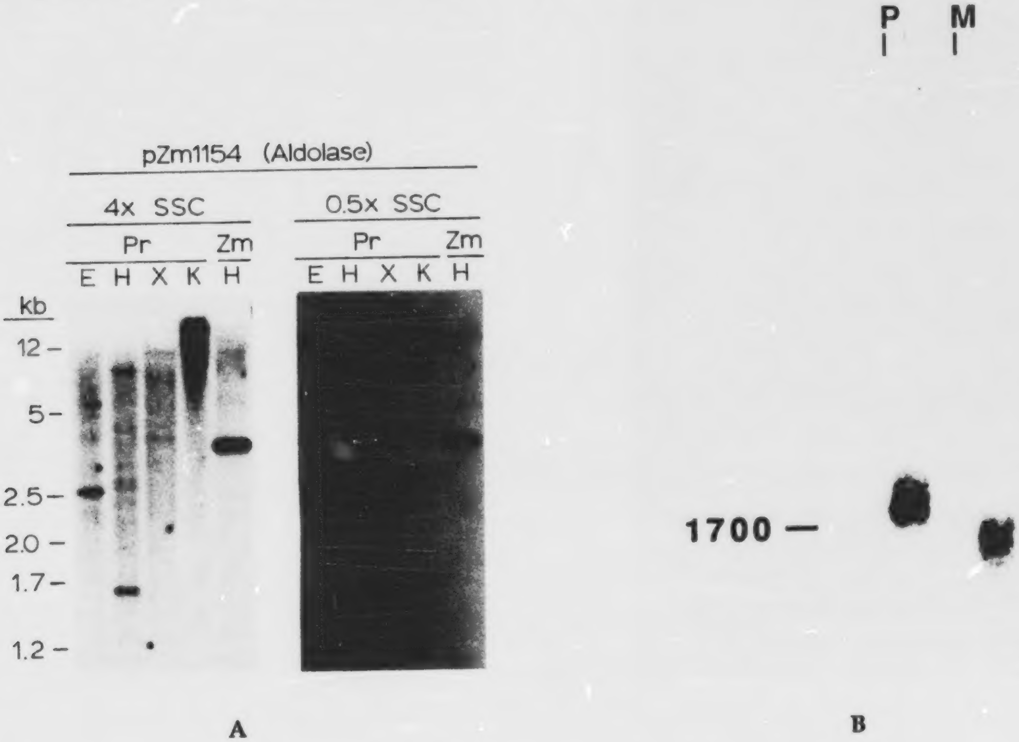


Figure 2. Test of hybridization stringencies

A. Genomic Southern of aldolase - Genomic DNA was digested with various restriction enzymes, eg., E=*EcoRI*, H=*HindIII*, X=*XbaI*, K=*KnpI*, G=*Bg1I*, fractionated electrophoretically, blotted onto nitrocellulose, and hybridized to the maize *Ald* (pZM1154) cDNA probe; the nitrocellulose filter was washed sequentially under two stringency conditions. Pr=Monterey Pine, Zm=maize

B. Northern of *Adh* - Poly(A)$^+$ RNA was fractionated electrophoretically through a denaturing agarose gel containing formaldehyde and transferred to a Hybond-N nylon membrane. RNA was hybridized to the maize *Adh*1 cDNA probe (pZML793) and the nitrocellulose washed in 4XSSC at 55°C. P = Monterey Pine, M=maize anaerobic root. 1700 indicates the position of marker rRNA mobility.

cDNA Synthesis and Cloning into λgt10

Starting with poly(A)$^+$ RNA isolated from Monterey pine suspension cells, we generated cDNAs of larger than 500 bp (Figure 3). A library of 5×10^5 clones was obtained from approximately 2 μg of this cDNA. All clones from this library were given the letters RSC (*radiata*, suspension, cDNA). When screened with the maize *Ald* probe, approximately 100 positive clones were identified and, of these, 11 were verified through a second round of screening. When screened with the maize *Adh*1 probe, 40 positive clones were identified of which seven were verified through a second round of screening.

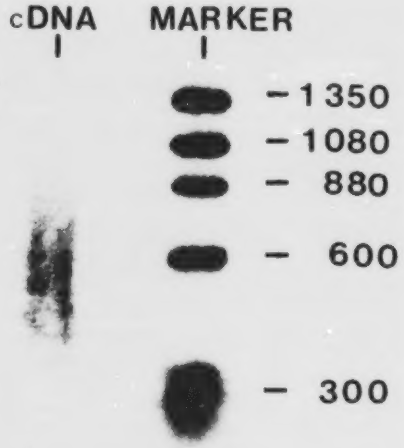


Figure 3. Final cDNA - Following size fractionation, the radiolabelled cDNA was fractionated electrophoretically through an alkaline denaturing gel and an autoradiogram of the gel developed. M=molecular weight standards.

Figure 4 shows a representative pair (A and B) of duplicate plaque hybridization filters from the first round of screening with the maize *Adh*1 cDNA probe. Autoradiogram signals for positive plaques, eg. RSC1016, varied. Selecting only plaques which hybridized to the *Adh*1 probe on both of the duplicate filters allowed for elimination of "false positives". Similar hybridization results were obtained when the library was screened for *Ald* cDNAs (data not shown).

Figure 5 shows a representative pair (A and B) of plaque hybridization filters from the second round of screening (for the RSC1016 clone shown in Figure 4) with the *Adh*1 probe. The signal intensities for these second round plaques were much greater than signal intensities from the corresponding first round plaques. These plaques were plated at a much lower density and each plaque is larger than those from the original plating of the library. Similar hybridiza-

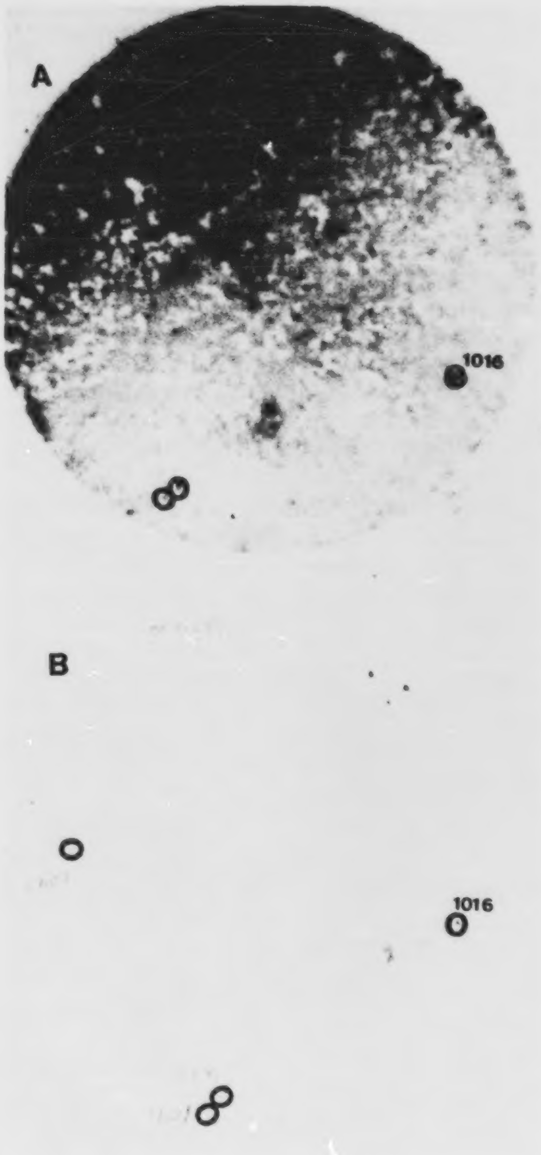


Figure 4. First round of library screening - Duplicate filters (A and B) from each petri dish of the library were hybridized to the maize *Adh*1 cDNA probe (pZML793).

tion results were obtained for the second round of screening *Ald* clones. The *Ald* clones have not been analyzed further.

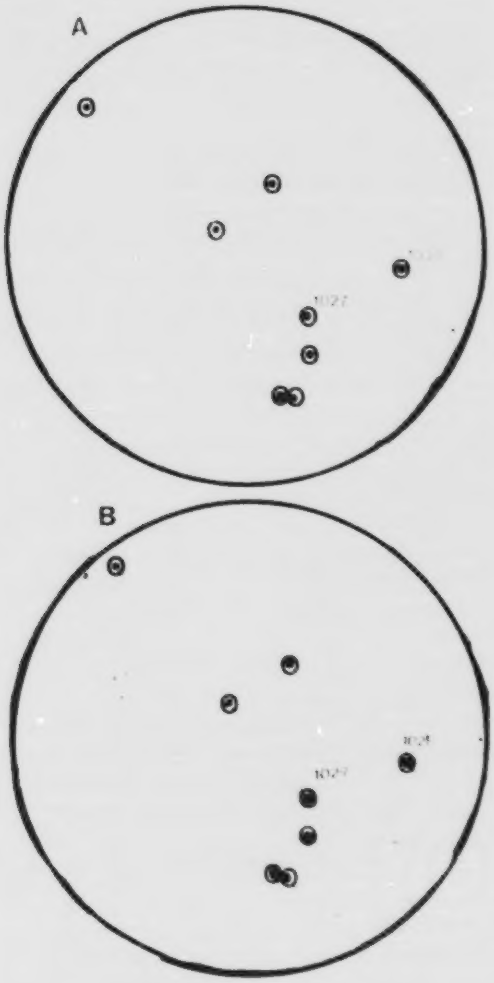


Figure 5. Second round of library screening - Duplicate filters (A and B) from each petri dish of the replated plaques were hybridized to the maize *Adh* cDNA probe (pZML793).

Characterization of individual *Adh* Clones

DNA from five individual *Adh* clones was isolated and digested with *Eco*RI to determine the sizes of each cDNA inset. Two of the clones contained inserts which were smaller than 200 bp; these were not studied further. Three of the clones, RSC1019, RSC1025, and RSC1027 all contained inserts of approximately 500 bp (eg. RSC1025, Figure 6). To verify that these cDNA clones carried *Adh* inserts, Southern blot hybridization was performed for clones RSC1019, RSC1025, and RSC1027 from gels similar to that shown in Figure 6. All three 500 bp *Eco*RI cDNA inserts hybridized to the maize *Adh* cDNA probe (eg. RSC1025, Figure 7).

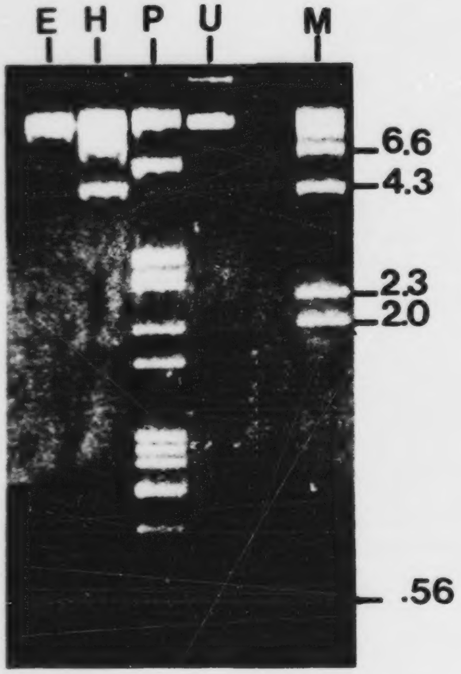


Figure 6. Restriction enzyme digestion of pine *Adh* clone - Lambda DNA for clone RSC1025 was digested with *Eco*RI (E), *Hind*III (H), and *Pst*I (P) and fractionated electrophoretically through a 1% agarose gel containing 50 µg/mL ethidium bromide. U=undigested DNA for clone RSC1025.

The DNA sequences of two of the cDNA clones, RSC1025 and RSC1019, were determined and will be published elsewhere. Clone RSC1025 begins near the ATG start codon of the *Adh* gene and extends approximately 500 bp into the *Adh* coding sequence. A dot matrix comparison between the DNA sequence of RSC1025 and the maize *Adh*1 cDNA shows that the two *Adh* sequences share approximately 70% homology (Figure 8).

Figure 7. Southern blot of the pine *Adh* clone - DNA in the gel in Figure 6 was transferred to Hybond-N, hybridized to the maize *Adh*1 probe (pZML793), and washed under the same stringency conditions used to screen the library, 4XSSC and 55°C.

These results demonstrate the feasibility of using heterologous probes to isolate and characterize genes from conifers whose DNA sequences have been evolutionarily conserved. Sequence analysis of additional cDNAs spanning the Monterey pine *Adh* RNA will allow the DNA and protein sequences of the entire coding region for a gymnosperm *Adh* to be determined. Comparison of angiosperm *Adh* cDNA sequences and gymnosperm *Adh* cDNA sequences will provide data for calculating genetic distances between these taxa. In addition to providing sequence information for evolutionary comparisons, the Monterey pine *Adh* cDNAs we have isolated will provide hybridization probes for isolating pine *Adh* genomic sequences and for studying the induction of pine *Adh* RNA during anaerobic stress.

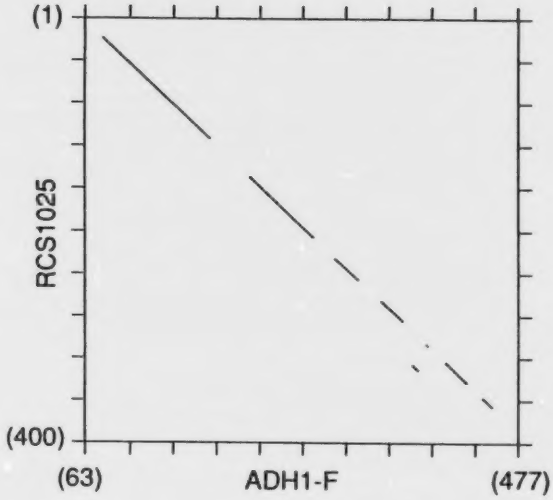


Figure 8. Homology matrix of maize and pine cDNA sequences - Using the DNA Inspector II homology matrix program, the maize *Adh*1F (pZML793) and pine RSC1025 DNA sequences were compared using 20 bp units and allowing 6 bp mismatches (30%).

Acknowledgments

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CLONING OF A cDNA ENCODING THE LIGHT-HARVESTING CHLOROPHYLL *A/B*-BINDING PROTEIN (*CAB*) FROM SCOTS PINE (*PINUS SYLVESTRIS* L.).

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Abstract

We describe a method to prepare mRNA from seedlings of Scots pine (*Pinus sylvestris* L.). Seedlings were grown in the dark for three weeks and exposed to light for up to three days before mRNA was extracted. A modification of the guanidinium isothiocyanate (GTC)/cesium chloride method was used. The method typically yielded 0.4 mg of RNA per g fresh weight. Northern hybridization to a pea cDNA clone, encoding the chlorophyll *a*/*b*-binding protein (*Cab*-gene), showed that the RNA was undergraded and that sequence homology exists between the pea and pine species. A prominent mRNA, 1100 nucleotides long, was detected. A cDNA library was constructed in λgt10 and was screened with the pea *Cab*-gene as probe. Several positive clones were isolated. The identity of one clone was confirmed by DNA sequencing. The DNA sequence of the confirmed *cab*-gene from Scots pine shows a homology between 83% and 97% compared to other sequenced *Cab*-genes.

Introduction

Pines (genus *Pinus*) which are gymnosperms, often dominate northern hemisphere forest ecosystems. Gymnosperms differ physiologically from angiosperms in a number of ways. One fundamental difference is their ability to form substantial amounts of chlorophyll in the absence of light (reviewed in Castelfranco 1983). In dark-grown pine tissue chloroplasts are well-developed compared with angiosperms under the same conditions, and both chlorophyll *a* and *b* are present. (The amount of these chlorophylls, however, is less than 10% of that if the tissue is grown in light for six days). Chlorophyll *b* is also incorporated into the light-harvesting complex of photosystem II (LHC-II) in the dark. Thus, there appear to be fundamental differences in the light regulatory framework of photosynthesis between angiosperms and gymnosperms.

The chlorophyll *a*/*b*-binding apoproteins of LHC-II are encoded by a gene family in all plants (all angiosperms, so far) studied (Coruzzi et al. 1983, Dunsmuir et al. 1983, Dunsmuir 1985, Karlin-Neumann 1985, Lamppa et al. 1985, Pichersky et al. 1985, Sheen and Bogorad 1986). The LHC-II is composed of different polypeptides of slightly different molecular weight (Thornber et al. 1986). The major difference seems to be in the N-terminal part, which is involved in the lateral movement of the complex (Mullet 1983). Most of the published *Cab*-genes seem to belong to the same subfamily, although a few divergent genes which encode slightly smaller mature polypeptides have been characterized (Karlin-Neumann et al. 1985, Smeekens et al. 1986, Stayton et al. 1986). The cab-genes are light-regulated; the proteins and their mRNA are only present in low amounts in dark-grown material (Batchauer and Apel 1984, Cuming and Bennett 1981, Nelson et al. 1984, Pichersky et al. 1985, Silverthorne and Tobin 1984, Slovin and Tobin 1982, Thompson et al. 1983, Viro and Kloppstech 1982, Zhu et al. 1985). In addition, the light-induced accumulation of *Cab*-transcripts in barley, for example, requires development of the chloroplast (Batschauer et al. 1986). The *CAB* protein is involved in many interactions with other proteins and pigments in the photosynthetic apparatus and the coding part of the *Cab*-gene could, therefore, be assumed to have a high degree of conservation during evolution. This raised the possibility that the *Cab*-gene from pine could be cloned using a *Cab*-gene from pea as the probe.

We know little about the molecular genetics of pines. Although preparation of nuclear DNA (Gustafsson and Sitbon 1986) and chloroplast DNA (White 1986) has been reported, no method for preparing high-quality mRNA, suitable for cDNA cloning, has been published.

In this paper we report the preparation of mRNA from Scots pine (*Pinus sylvestris* L.), and cloning of the *Cab*-gene from a cDNA library. This permitted the study of the structure and function of the isolated *Cab*-gene.

Materials and Methods

Plant material

Seeds of Scots pine (*Pinus sylvestris* L.) were germinated and seedlings grown in absolute darkness for three weeks at 23°C. Prior to harvest, plants were exposed to continuous white light for different time periods. Cotyledons and stems were then harvested. All plant material was placed in liquid nitrogen after harvesting and stored at -80°C.

Plasmids

A 850 bp *Pst*I-fragment from plasmid pAB96, containing part of the coding sequence for a *Cab*-gene from pea, was used (Coruzzi et al. 1983). Plasmid pUC19 was used for DNA-sequencing (Yanisch-Perron et al. 1985).

RNA-extraction

A modification of the standard guanidium isothiocyanate (GTC)/cesium chloride (CsCl) method was used to prepare RNA (Maniatis et al. 1982). The material was frozen in liquid nitrogen, ground in a Braun coffee-mill, and transferred to GTC buffer (4 M guanidium isothiocyanate, 50 mM Tris-HCl, pH 7.6, 4 mM disodium EDTA, 2% sodium lauryl sarcosine, 1% β-mercaptoethanol). Cellular debris were removed by centrifugation in a Beckman J2-21 centrifuge at 6000 rpm for 5 min using a JA20 rotor. CsCl was added to the clear supernatant to a final concentration of 2.4 M and centrifuged in a Beckman JA20 rotor at 16 000 rpm for 10 min. The supernatant was layered onto a 5.7 M CsCl-cushion and centrifuged for 18 h at 34 000 rpm in an SW41 rotor. The supernatant was removed, the RNA pellet dissolved in RNA-buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1% sodium dodecylsulphate (SDS)), phenol/chloroform extracted, and ethanol precipitated twice. RNA concentration was measured on a Shimadzu spectrophotometer. RNA preparations were run on 1% agarose gels in 0.01 M phosphate buffer (pH 7.0) to check the quality of RNA. As a control, RNA was prepared from spinach using the same method.

Oligo-dT-chromatography

Oligo-dT-cellulose (Type 3, Collaborative Research) was swollen and packed in 2 mL disposable Econocolumns (BioRad). The denatured RNA sample was loaded on the column in application buffer (0.5 M NaCl, 1 mM EDTA, 0.1% SDS) and the poly(A)$^+$ RNA was eluted with elution buffer (1 mM EDTA, pH 7.5, 0.5% SDS) heated to 37°C and recovered by ethanol precipitation. Two cycles of oligo-dT-chromatography were performed.

Northern blot

Northern blot analysis was performed using formaldehyde gels (Maniatis et al. 1982). The hybridization probes were labelled with ^{32}P by Amersham's Multi-prime labelling system to a specific activity exceeding 10^9cpm/µg DNA.

Construction of the cDNA-library

cDNA was synthesized from 5 µg poly(A)$^+$ RNA extracted from plants exposed to white light for three days, using a cDNA synthesis kit from Amersham. The reaction conditions were as recommended by the manufacturer. The blunt-ended cDNA was *Eco*RI-methylated. *Eco*RI linkers were ligated onto methylated cDNA, after which the mixture was treated with *Eco*RI using standard procedures. The pine cDNA was ligated to *Eco*RI-digested, dephosphorylated, λgt10-arms (Stratagene) in equal molar amounts, assuming that the average length of the cDNA was 600 bp. Ligation was carried out for 18 h at 15°C and added to a λ *in vitro* packaging mixture. *In vitro* packaging extract (Gigapack Plus, Stratagene) was handled according to the manufacturer's instructions using λ-competent *E. coli* cells, strains C600 and C600Hfl.

Screening the cDNA-library

Plaque-blotting onto Hybond-N-membrane was performed following the Hybond manual. The probes were labelled as described in Northern blot section. Hybridization was performed with the pAB96-probe for 17 h at 55°C in 6X SSC (1X SSC = 0.15 M NaCl, 0.015 M trisodiumcitrate), 5X Denhardt, 0.5% SDS, 9% dextran sulphate, and 100 μg/mL denatured herring sperm DNA. The membranes were washed in 2X SSC for 15 min, 2X SSC, 0.1% SDS for an additional 15 min, and finally in 1X SSC, 0.1% SDS for 15 min. All washings were performed at 55°C. The membranes were dried briefly and autoradiographed using Cronex 4 film. Putative positive plaques were picked with a Pasteur pipette and plated, blotted, and hybridized as above.

DNA-sequencing

DNA isolated from positive plaques were cut with *Eco*RI and recloned into pUC19 (Yanisch-Perron et al. 1985). The plate lysate method for rapid, small-scale isolation of λ DNA was used and the DNA was digested and analyzed on agarose gels using standard techniques (Maniatis et al. 1982). DNA sequencing was performed with the dideoxy chain termination method (Sanger et al. 1977). The DNA was analyzed using GENEUS software (Harr et al. 1986).

Results

Preparation of mRNA from pine seedlings

Seedings yielded 0.2 to 0.5 mg RNA per gram fresh weight when the described method was used. RNA could be extracted from 10 to 15 g of material without any sign of degradation visible on agarose gel. Exposure of seedlings to light up to three days before harvest did not reduce yield. After one cycle of oligo-dT-chromatography, 1.8% to 2.5% of the RNA was recovered as poly(A)$^+$ RNA and, after two rounds, 0.6%. *In vitro* translation experiments using wheat germ extracts indicated that a great portion of translatable RNA eluted with the poly-(A)$^+$ fraction as well.

The *Cab*-gene from Scots pine shares homology with its pea counterpart.

The isolated RNA was separated on agarose gels, subjected to Northern hybridization, and hybridized to a *Cab*-gene from pea. A purified *Cab*-fragment was used because earlier experiments in our laboratory showed that the cloning vector pBR322 hybridizes unspecifically to pine genomic DNA (data not shown). As seen in Figure 1, lane 3, a prominent RNA species from Scots pine hybridized to the heterologous *Cab*-fragment, although the hybridization signal was weaker compared to that of spinach RNA run as a control (Figure 1, lane 2). The result

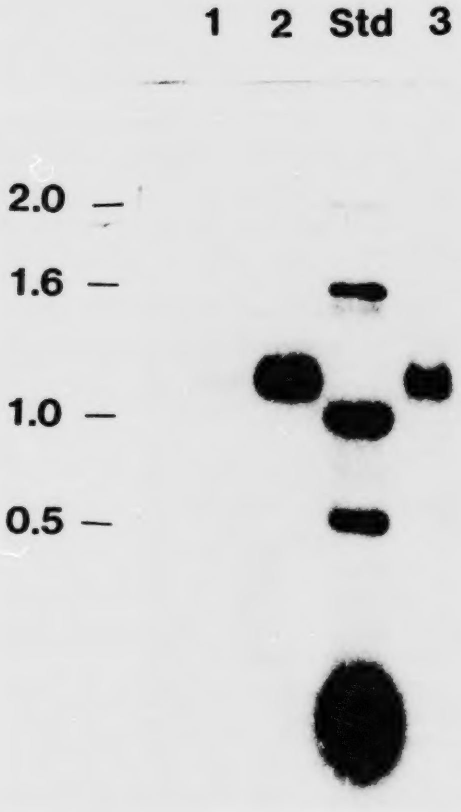

Figure 1. Northern blot of a *Cab*-gene from pea and RNA isolated from Scots pine and from spinach. Lane 1: 0.6 μg of poly(A)$^+$RNA from pine seedlings grown in the dark for 14 days. Lane 2: 0.6 μg of poly(A)$^+$RNA from spinach grown in the dark for 12 days and transferred to light for an additional two days. Lane 3: 6 μg of total RNA from pine seedlings treated as in Lane 2. Std: Molecular weight standard. Sizes of the molecular weight standard are shown at left.

shows that the nucleotide sequence homology is probably lower between pine/pea than spinach/pea. The size of the transcript in pine and spinach is equal (about 1100 bases).

Cloning the *Cab*-gene from Scots pine

As estimated by TCA precipitation, 5% to 7% of the RNA was reverse transcribed into cDNA and cDNA was converted almost completely into dsDNA. The subsequent steps prior to ligation to λgt10 arms resulted in a loss of about 75% of the cDNA. One packaging reaction resulted in 400 000 pfu/reaction, of which the vast majority formed plaques on strain C600Hfl, indicating that these plaques contained inserts.

A portion of the library (30 000 plaques) was screened with the purified *Pst*I-fragment containing part of the *Cab*-gene from pea. A large number of plaques hybridized to the probe. One clone, CAB4, was chosen for further study.

DNA sequencing of the *Cab*-gene from the *CAB4*-clone

DNA isolated from the *CAB4* clone was cut with *Eco*RI and ligated into plasmid pUC19. The recombinant pUC19 plasmid was DNA sequenced using the dideoxy chain termination method. The resulting DNA sequence is shown in Figure 2. The DNA sequence was translated into amino acids using the GENEUS computer package (Harr et al. 1986). The obtained amino acid sequence was compared to other *Cab* sequences from previously cloned and sequenced *Cab*-genes. The result is shown in Figure 3. It is clearly seen that the *CAB4* amino acid sequence is highly homologous to other *Cab* sequences and an inference can be safely made that this clone is a *Cab*-gene from Scots pine.

```
 1                             10                              20
AspAspProGluAlaLysAlaGluLeuLysValLysGluIleLysAsnGlyArgLeuAla
GACGACCCCGAGGCCAAGGCGGAGCTGAAGGTGAAGGAGATTAAGAACGGTCGGCTGGCC
1       10        20        30        40        50        60

                               30                              40
MetPheSerMetPheGlyPhePheValGlnAlaIleValThrGlyLysGlyProIleGlu
ATGTTCTCCATGTTCGGTTTCTTCGTTCAGGCAATCGTGACCGGGAAGGGCCCCATTGAA
        70        80        90       100       110       120

                               50                              60
AsnLeuTyrAspHisLeuAlaAspProValAlaAsnAsnAlaTrpAlaTyrAlaThrAsn
AATCTCTACGACCACTTGGCGGACCCCGTTGCCAACAATGCCTGGGCCTACGCCACCAAT
       130       140       150       160       170       180

PheValProGlyLys***
TTCGTTCCTGGCAAGTGA
       190
```

Figure 2. Partial DNA and amino acid sequence from the *CAB4* gene cloned from Scots pine. The last 197 bases and 65 amino acids in the chlorophyll *a*/*b*-binding gene and protein, respectively, are shown.

```
                  1       10        20        30        40        50        60
Scots pine CAB4   DDPEAKAELKVKEIKNGRLAMFSMFGFFVQAIVTGKGPIENLYDHLADPVANNAWAYATNFVPGK
                    * * *** ******************* ********   * ** **** ****  ********

Pea CAB15         EVPEAFAELKVKELKNGRLAMFSMFGFFVPAIVTGKGPLENLADHLADPVNNNAWSYATNFVPGK
Petunia CAB13     DDPEAFAELEVKEIKNGRLAMFSMFGFFVQAIVTGKGPLENLADHLADPVNNNAWAFATNFVPGK
Petunia CAB37/IB  DDPEAFAELKVKEIKNGRLAMFSMFGFFVQAIVTGKGPIENLYDHVADPVANNAWAFATNFVPGK
Wheat AB1.6       DRPQAFAELKVKEIKNGRLAMFSMFGFFVQAIVTGKGPLGDLADHIADPVNNNAWLIATNFVPGK
Tomato CAB 1A     EDPEAFAELKVKEIKNGRLAMFSMFGFFVQAIVTGKGPLENLADHLADPVNNNAWAFATNFVPGK
Lemna pLgAB19     DDPEAFAELKVKEIKNGRLAMFSMFGFFVQAIVTGKGPIENLADHIADPVANNAWAFATNFVPGK
                    * * *** ******************* ********   * ** **** ****  ********
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Figure 3. Comparison of the amino acid sequences between the *CAB4* gene of Scots pine (derived from the DNA sequence) and the chlorophyll *a/b*-binding protein of six angiosperms. The last 65 amino acids in the protein are shown. * represents positions where the seven sequences are identical. The following sequences were used: pea *CAB*15 (5), petunia *CAB*13 (7), petunia *CAB*37/IB (23), wheat AB1.6 (12), tomato *CAB*1A (17), and *Lemna* pLgAB19 (11).

	Pea *CAB*15	Pet *CAB*13	Pet *CAB*37	Wh AB1.6	Tomato 1A	*Lemna* AB19
Scots pine *CAB*4	8	6	3	11	6	4
Pea *CAB*15		6	9	9	4	8
Petunia *CAB*13			6	8	2	4
Petunia *CAB*37				10	6	2
Wheat AB1.6					8	8
Tomato 1A						5

Figure 4. Levels of homology between the *CAB4* amino acid sequence from Scots pine and the chlorophyll *a/b*-binding protein from six angiosperms. The last 65 amino acids were compared. The amino acid sequences were compared pairwise. The figures show the number of non-identical amino acids. The following amino acid sequences were used: pea *CAB*15 (5), petunia *CAB*13 (7), petunia *CAB*37/IB (23), wheat AB1.6 (12), tomato *CAB*1A (17), *Lemna* pLgAB19 (11).

The comparison between the *CAB4* amino acid sequence from Scots pine and the chlorophyll *a/b*-binding proteins from other plants revealed a high degree of conservation between angiosperms and gymnosperms (Figure 4). The following *Cab*-sequences were used: pea *CAB*15 (Coruzzi et al. 1983), petunia *CAB*13 and *CAB*37/IB (Dunsmuir et al. 1983, Stayton et al. 1986), wheat AB1.6 (Lamppa et al. 1985), tomato *CAB* 1A (Pichersky et al. 1985), and *Lemna* pLgAB19 (Karlin-Neumann et al. 1985). The overall DNA and amino acid sequence homology ranges between 75 to 87% and 83 to 95%. respectively. The level of homology between the pine *Cab* sequence and the angiosperm sequences was not lower than the homology among the different angiosperm species. The result shows that the *Cab*-gene has been well conserved through evolution.

Discussion

In general, preparation of macromolecules in general from pines is difficult, mainly because of the large amounts of phenolic compounds and other secondary metabolites in pine tissue. When needles were used (data not shown) for RNA preparation, no intact RNA could be recovered. However, these difficulties can be largely avoided by using dark-grown seedlings. The RNA prepared from seedlings was of a high quality. The mRNA was undegraded, translatable *in vitro*, and could be used as a substrate for the AMV reverse transcriptase in initial reactions for synthesizing a cDNA library.

The genes coding for the chlorophyll *a/b*-binding proteins have so far been found as a gene family in all previous studies. Preliminary Southern blot experiments using total genomic DNA from Scots pine and the *CAB4* clone presented in this communication indicates that the *Cab*-gene is present as a gene family in pines as well. A common feature of most *Cab*-genes analyzed so far has been the absence of introns. Exceptions to the rule have been found in *Lemna* and petunia (Karlin-Neumann et al. 1985, Stayton et al. 1986). We cannot say whether or not pines lack introns from Northern blot experiments performed with pine RNA. Further experiments using homologous probes in Northern blot hybridizations, DNA cloning, and analysis of genomic *Cab*-genes have to be made to determine the genetic organization of these genes in pines.

The high level of homology between *Cab* DNA and the amino acid sequence from angiosperms and gymnosperms indicate a well preserved function of the chlorophyll *a/b*-binding protein. The result raises the question as to whether other highly conserved genes could be used as heterlogous probes to isolate pine genes. Parallel to the pea *Cab*-gene, we used a gene coding for the small subunit of Rubisco from pea (Bedbrook et al. 1980) as a probe in Northern blot experiments and when screening the cDNA bank. In contrast to the *Cab*-gene, no signal could be detected when using the *ssu*-probe. The result indicates that the level of homology between the *ssu*-genes from pine and pea is lower than that found for the *Cab*-gene.

The method developed to isolate RNA from pine seedlings was also tested on mature pine tissue. No RNA could be detected in the isolations. It is apparent that new methods, or at least modifications of the old, have to be used to isolate RNA from pines that are older than seedlings.

We have described the successful isolation of RNA from pines and the cloning of genes coding for the chlorophyll *a/b*-binding protein from Scots pine. We hope to use the isolated *Cab*-gene to study the family of *Cab*-genes in pines as well as the transcriptional regulation of its mRNA.

Acknowledgments

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MOLECULAR GENETIC ANALYSIS OF DEVELOPMENT AND MATURATION IN LARCH

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Abstract

Maturation in conifers is a developmental process resulting in reproductive competence and a reduction in i) the growth rate, ii) the rootability of cuttings, and iii) the potential for propagating tissue in culture. To understand the genetic mechanisms governing the switch from a juvenile to a mature form of growth, we have constructed recombinant DNA libraries from RNA expressed in the short shoots of larch *Larix laricina* (Du Roi) K. Koch. Using DNA hybridization techniques, we identified clones which were apparently expressed in a differential manner between juvenile and mature trees. Subsequent analysis of some of these clones suggested that the differential signal was the result of genetic background and not maturation. In addition, we have identified clones representing RNA transcripts encoding the small subunit of ribulose-1,5-bisphosphate carboxylase. Using Northern blot analysis, a transcript of approximately 1000 nucleotides (nt) has been identified, which is expressed in foliage but not in roots.

Introduction

Maturation, or phase change, in the conifers is a developmental process that is poorly understood at present. It is exemplified by such characteristics as a reduced rate of growth, reduced efficiency in the rooting of cuttings, changes in foliar morphology, and the onset of flowering. Maturation and its reversal are of interest for several reasons. The rapid growth of the juvenile phase is essential for the young tree to compete with other vegetation. Prolonging the juvenile phase may be of use in the production of biomass from conifer forests. Alternatively, shortening the juvenile phase would be of use in accelerated breeding programs for the production of genetically improved trees. The ability to reverse maturation will also be important for the propagation of specific mature trees via either rooted cuttings or tissue culture. Finally, an understanding of the maturation process is necessary to assure that any rejuvenation achieved is followed by a normal maturation sequence (Greenwood 1987).

The events which may regulate maturation are not known. Hackett (1985) discussed whether the stability of the mature phase was determined at the level of the individual cell or the entire apical meristem, or was due to correlative effects involving the whole plant. Greenwood's data (Greenwood 1984, Greenwood et al., in prep.) suggests maturation is a result of permanent genetic changes within the meristem of the developing conifer, rather than a consequence of the increased size and/or structural complexity of the plant, as suggested by others (Borchart 1975, Durzan 1984).

It is our working hypothesis that maturation is the result of genetic changes in the meristem of the tree, and that the different phenotypes of the mature and juvenile phases will be reflected in the RNA transcripts found in the respective plants. We have initiated a study to detect genes which are differentially expressed in juvenile and mature trees using the conifer *Larix laricina* (larch) as a model system. Complicating the study is the fact that conifers, including larch, are highly outbred, with diverse genetic backgrounds which may affect the expression of individual genes (e.g., Cheliak and Pitel 1985, Fins and Seeb 1986).

Materials and methods

Construction of grafted larch scions. Scions were taken from vigorously growing branches in the upper one-third of the crown of four sexually mature field-grown trees ranging 13 to 25 years in age (tree numbers 11, 12, 14, 15). Simultaneously, scions were taken from the main stem of one-year-old seedlings grown from open pollinated progeny of the first four trees. In order to standardize scion diameter and minimize possible topophytic effects, the scions were pruned down to 20 cm stem segments, and the terminal bud was always removed. Thus, all shoots emerging after grafting arose from lateral buds. All scions were side-grafted onto uniform 2-year-old larch rootstock.

Extraction of RNA and construction of cDNA clones. RNA was extracted from expanding short shoots (ca. 2 cm long) consisting of elongating needles, leaf primordia, and the meristem. RNA was extracted as described by Whitmore and Kreibel (1987), with the exception that 0.1% Triton X-100 (Boehringer Mannheim) was substituted for SDS in all phases of the procedure. Poly(A)$^+$ RNA was purified using poly-U mAP filter paper (Amersham) as described by the supplier, except that the RNA was preincubated with the mAP paper for 5 min at room temperature before being blotted through the filter.

The cDNA was synthesized via a modification of the method of Gubler and Hoffman (1983). The reaction mixture (20 µL) contained: Tris-HCl, 50 mM (pH 8.3); KCl, 75 mM; dithiothreitol (DTT), 10 mM; $MgCl_2$, 3 mM; dATP,dCTP,dGTP, TTP, 1 mM each; random hexamer primers (Pharmacia), 250 µg/mL; RNA, 1 µg; and cloned Moloney MLV reverse transcriptase (BRL), 200 U. The reaction was incubated at 37°C for 1 h to synthesize the first strand. For second strand synthesis the entire reaction was diluted to 100 µl with buffer and dH_2O to maintain the concentrations of the Tris, KCL, DTT, and $MgCl_2$. RNase H, 0.8 U (Amersham) and *E. coli* DNA polymerase I, 23 U (New England Biolabs) were added and the reaction incubated at 12°C for 1 h, and then at 22°C for 1 h. The reaction was stopped by heating to 70°C for 10min. The ends of the cDNA were "polished" by the addition of T4 DNA polymerase, 2 U (Promega), and incubation for 10 min at 37°C.

The cDNA was treated with *Eco*RI methylase (Promega), and *Eco*RI linkers (New England Biolabs) were ligated onto the ends. Complementary DNA of greater than 500 bp was size-selected on agarose gels and cloned into the vector λgt10. Typical libraries contained 1 to 4 X 10^6 plaques.

Screening of the cDNA libraries. Two to four thousand plaques from each cDNA library (juvenile and mature) were plated at a density of 300 plaques in a 15 X 100 mm petri dish. The phage were plated on LB agar using *E. coli* C600 *Hfl* as a host. Plaque lifts were performed as described by Benton and Davis (1977), except that Hybond nylon filters (Amersham) were used. The DNA was fixed to the filter using short wavelength uv light. The filters were probed with ^{32}P-labelled cDNA prepared from either juvenile or mature poly(A)$^+$ RNA. Each filter was probed with 5 x 10^6 cpm/mL of probe. To isolate clones representing the small subunit of Rubisco, we labelled a clone for the homologous sequence from maize using the random primer labeling method of Feinberg and Vogelstein (1983).

Hybridization conditions for the plaque lifts were as described by Church and Gilbert (1984), except that the Rubisco probe was washed in $NaPO_4$, pH 7.2, 0.5 M; EDTA, 1 mM; and SDS, 0.1%. This was necessary to reduce the stringency of the wash. DNA sequencing was performed as previously described (Hutchison, et al., 1984) except that the reactions were run at 50°C. For the Northern blots RNA was electrophoresed in an agarose-formaldehyde gel as described by Maniatis et al. (1982), and blotted onto Zetabind nylon filters (AMF Cuno) with 20 X SSC. The RNA was fixed to the filters with uv light.

The Northern blots were probed with ^{32}P-labeled DNA in a buffer containing, 4 X SSC; Na_2PO_4, pH 7.2, 50 mM; 1 X Denhardt's solution (Denhardt 1966); poly-A, poly-C (Sigma), 2.5 mg/mL of each; sonicated salmon sperm, 5mg/mL; deionized formamide (BRL), 50%; and 1-5 x 10^6 cpm/mL of probe. The hybridization temperature was 42°C. The filters were washed in 2 X SSC, 0.1% SDS at 50°C.

Results

Purification of RNA from larch. Ten grams wet-weight of tissue gave approximately 5 mg of total RNA of which 0.3 to 0.5% was purified as poly(A)$^+$ RNA. The yield of both total and poly(A)$^+$ RNA was essentially the same for both mature and juvenile trees.

Before substitution of the non-ionic detergent Triton-X100 for SDS in the extraction protocol, there was a significant sub-260 nm component to the spectrum, especially for the poly(A)$^+$ RNA. A small amount of the contaminant still remained even with the use of Triton-X100. We have not yet attempted to use other non-ionic detergents. We do not know if this contaminant interferes with efficiency of poly(A)$^+$ selection. However, we do know the contaminant interferes with oligo-dT primed cDNA synthesis (data not shown).

Identification of clones which are expressed in a maturation-related manner. To screen for clones which may represent sequences that are expressed differentially between juvenile and mature trees, cDNA libraries from RNA extracted from meristematic tissue of juvenile and mature trees were screened with radioactively labelled cDNA representing either juvenile or mature tree transcripts. Representative results are shown in Figure 1. A large number of plaques showing differential hybridization were seen, of which 43 were picked. Some of these clones showed a stronger hybridization to a cDNA probe from RNA of mature plants, and some clones showed a stronger hybridization to a cDNA probe from RNA of juvenile plants. No clones were detected which were expressed only in mature plants or only in juvenile plants.

Six clones were analyzed further. DNA from each of these were prepared and labelled with ^{32}P, and then hybridized to Northern blots containing RNA from juvenile and mature plants of three different tree families. The results with one of these clones is shown in Figure 2. The initial screen used RNA from juvenile and mature plants in family 11 for synthesizing the cDNA probe. The differential hybridization was confirmed on the Northern blot (Figure 2, lanes 11M and 11J). However, this difference was not seen in the RNA from other tree families (Figure 2, lanes 12M, 12J, 15M, and 15J). Similar results were found for the other clones analyzed (data not shown), and were independent of whether the clone represented a sequence which was ex-

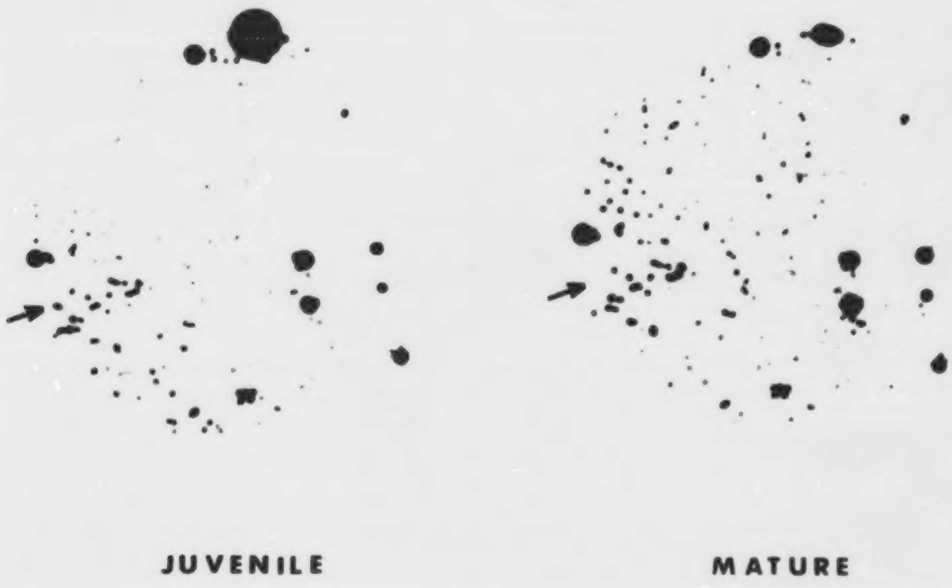

Figure 1. Hybridization of juvenile and mature probes to duplicate plaque lifts of a larch cDNA library. Four plaque lifts were made from each plate of clones from the mature and juvenile cDNA libraries. Two of the lifts were hybridized to a probe made from mature tree RNA and two were hybridized to a probe made from juvenile tree RNA. A typical plate is shown and a differentially hybridizing plaque is indicated by the arrow. Mature: Filter hybridized with a cDNA probe from mature tree RNA. Juvenile: Filter hybridized with a cDNA probe from juvenile tree RNA. Only plaques which hybridized differentially on both copies of the duplicate filters were taken for further analysis.

pressed at a higher level in juvenile plants or mature plants.

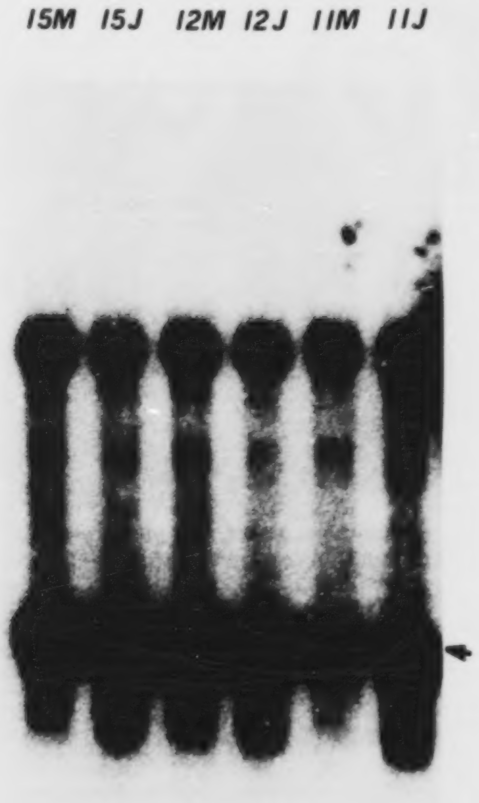

Figure 2. Northern blot of larch total RNA hybridized to a larch differential probe. Clone pCLDJ1.1 was originally identified as hybridizing more strongly to a juvenile probe from tree family #11 than to a mature probe from the same family. Two micrograms of total RNA from a juvenile and mature tree of three tree families (11, 12, and 15) were electrophoresed in a 1.2% agarose-formaldehyde gel and blotted onto a nylon filter. The filter was probed with ^{32}P-labelled pCLDJ1.1 and the filter exposed to Kodak X-Omat AR5 film. Lanes: **15M**, Total RNA from a grafter scion of tree 15, a mature tree, and a progenitor for family 15; **15J**, Total RNA from a 1-year-old grafted seedling which is an open pollinated progeny of tree 15; **12M, 12J, 11M, 11J**, The same as described for 15M and 15J, except representing mature and juvenile members of tree families 12 and 11, respectively.

Identification and characterization of small subunit-Rubisco sequences. As part of our analysis of the integrity of these libraries, we screened approximately 2000 plaques with a small subunit (ssu)-Rubisco (*rbcS*) clone from maize (obtained from W. Taylor, UC, Berkeley). Initial screening at a low stringency gave eight potential positive plaques. Subsequent rounds of purification of the plaques resulted in four clones that showed significant hybridization to the maize *rbcS* sequence.

Two clones, pCLRu1 and pCLRu2, were further characterized; pCLRu1 had an insert of 125 bp and pCLRu2 had an insert of 170 bp. Both have now been sequenced using the dideoxy chain termination method of Sanger et al. (1977), and the results are shown in Figure 3. Shown also is the sequence from a soybean *rbcS* clone (Berry-Lowe et al. 1982). As can be seen there is a significant degree of homology between the two sequences. The data suggest that the 3′ end of clone pCLRu1 is within one nucleotide of the 5′ end of clone pCLRu2, and there has been the insertion or deletion of 6 bp. We recognize that the mismatch is a potential cloning artifact and confirmation of this sequence awaits the analysis of genomic clones.

Alignment of the derived amino acid with other published amino acid sequences for the ssu-Rubisco is also shown. The primary significance of the sequence information in Figure 3 is that it confirms the clones are indeed representatives of *rbcS* transcripts. Based on this limited sampling there is no obvious greater similarity of the larch sequence to either the monocots or the dicots. In most regions of the sequence the larch ssu-Rubisco showed a strong conservation with angiosperm sequences.

Hybridization of the larch ssu-Rubisco probe to a Northern blot showed a transcript of approximately 1000 nt (Figure 4). This transcript was expressed in foliage but not in roots. It also showed no differential hybridization to RNAs from juvenile and mature plants.

Discussion

Maturation in conifers results in changes in the rate of growth, foliar morphology, and sexual and somatic reproductive capacities of the plant. These changes are essentially permanent, and are associated with the meristematic tissue (Greenwood 1984, Greenwood et al. in prep.). To analyze these events at the molecular level we cloned cDNA copies of RNA transcripts

Partial DNA and Amino Acid Sequence for Larch ssu-Rubisco.

A

DNA SEQUENCE

```
                    10        20        30        40        50        60
                     •         •         •         •         •         •
Larch       CTACGACGGGAGATACTGGGTGATGTGGAAGCTGCCTATGTTCGGATGCACAGAGGCATC
            ||| || ||  | ||||||   |||||||||||||||||||| || ||||| || || ||
Soybean     CTATGATGGACGCTACTGGACCATGTGGAAGCTGCCTATGTTTGGCTGCACTGATGCTTC
                     •         •         •         •         •         •
                  1125      1135      1145      1155      1155      1175

                    70        80        90       100       110       120
                     •         •         •         •         •         •
Larch       GCAGGTGTTGAACGAGGTGAACGACTGTGCGAAGGCATACCCCAACGCCTTCATCCGCC?
             ||||||||||| ||| |  | |||  |  ||  ||||||||||| | |||||||||
Soybean     TCAGGTGTTGAAGGAGCTTCAAGAGGCTAAGACTGCATACCCCAATGGCTTCATCCGT--
                     •         •         •         •         •
                  1185      1195      1205      1215      1225

                   130       140       150       160       170       180
                     •         •         •         •         •         •
Larch       ATCCGTCATCGGATTCGACAACGTCCGCCAAGTGCAGTGCATCTCCTTCATCGTCCACAA
            |||    || ||||| |||||||| ||||||||||||||||||  |||||||| | ||||
Soybean     ATC----ATTGGATTTGACAACGTTCGCCAAGTGCAGTGCATCAGCTTCATCGCCTACAA
             •             •         •         •         •         •
          1235          1245      1255      1265      1275      1285

                   190       200       210       220       230       240
                     •         •         •         •         •         •
Larch       GCCTGAATACAATTAAACTTGCAGCTCAATCTATTTTTCTGCGTCTCTTCTTCTTCCTCC
            ||| | |  |   |||       |     |  |             || |    |
Soybean     GCCCCCAGGCTTCTAAGTCATTNGAAGTTTGCAAAGCATCTGTAGCCTCCCCACTTTGTT
                 •         •         •         •         •         •
              1295      1305      1315      1325      1335      1345
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B

AMINO ACID SEQUENCE

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Larch     YDGRYWVMWKLPMFGCTEASQVLNEVNECAKAYPNAFIR?SVIGFDNVRQVQCISFIVHKPEYN
Lemna     YDGRYWTMWKLPMFGCTDASQVIAEVEEAKKAYPEYFVR--IIGFDNKRQVQCISFIAYKPT
Pea       YDGRYWTMWKLPMFGTTDASQVLKELDEVVAAYPQAFVR--IIGFDNVRQVQCISFIAHTPESY
Petunia   YDGRYWTMWKLPMFGCTDATQVLGELQEAKKAYPNAGSG--IIGFDNVRQVQCISFIAYKPPGY
Soybean   YDGRYWTMWKLPMFGCTDASQVLKELQEAKTAYPNGFIR--IIGFDNVRQVQCISFIAYKPPGF
Spinach   YDGRYW---KLPMFGGTDPAQVVNEVEEVKKAYPDAFVR--FIGFDNKREVQCISFIAYKPAGY
Tobacco   YDGRYWTMWKLPMFGCTDATQVLAEVEEAKKAYPQAWIR--IIGRDNVRQVQCISFIAYKPEGY
Wheat     YDGRYWTMWKLPMFGCTDATQVLNEVEEVKKEYPDAYVR--VIGFDNLRQVQCVSFIAFRPPGCEESGKA
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Figure 3. DNA sequence for a portion of the larch ssu Rubisco gene. Clone pCLRu1 and pCLRu2 were sequenced and aligned to the published sequence for a soybean *rbcS* gene. A: Alignment of the DNA sequence; B: Alignment of the derived amino acid sequence (single letter code) of the larch ssu Rubisco with the same segment from other plants.

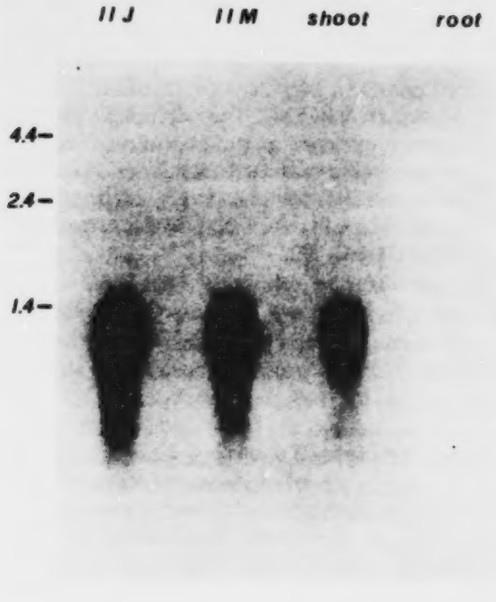

Figure 4. Northern blot of larch poly(A)$^+$ RNA hybridized with a larch ssu Rubisco probe. Two hundred nanograms of poly(A)$^+$ RNA were electrophoresed in a 1.2% agarose-formaldehyde gel and blotted onto a nylon filter. Lanes: **11J**, RNA from a juvenile tree from family #11; **11M**, RNA from the progenitor for family #11; **shoot**, RNA from the growing tips of a mixed sample of seedlings from tree family #11; **root**, RNA from the roots of the seedlings from tree family #11. Position and size (kilobases) of molecular weight standards.

from juvenile and mature plants of larch. Our initial approach was to use differential hybridization to detect sequences which are expressed in a maturation-related manner.

A large number of methods were tried to purify RNA from the meristematic tissue of larch. Of these, all techniques relying solely on the use of either phenolic or guanidinium compounds failed to extract RNA. This was true with or without the addition of RNAse inhibitors such as vanadyl ribonucleoside complexes or aurintricarboxylic acid. The addition of insoluble PVP (to remove phenolic resins) also had no effect. In fact, the addition of rat liver RNA to the extraction mixture resulted in a loss of the rat RNA. The only protocol we found to work was the RNA extraction protocol of Hall et al. (1978) as modified by Whitmore and Kreibel (1987). We have further modified the protocol by the substitution of 0.1% Triton-X 100 for SDS.

The yield of total RNA and poly(A)$^+$ was the same from both juvenile and mature plants. This suggests that maturation is not the result of a general decline in the level of transcription in meristematic tissue. Rogler and Dahmus (1974) similarly reported only minor differences in RNA production between the juvenile and mature phases of *Hedera helix*. Their data did, however, suggest that differences in the juvenile and mature phases were related to alterations in the rate of transcription of some genes in the apical meristem.

A significant sub-260 nm absorption was seen in the spectra of our RNA preparations; this absorption was reduced but not eliminated by the use of the non- ionic detergent Triton X-100. We believe this to be caused by a polysaccharide-containing contaminant similar to that reported by Takahashi and Nitta (1986), which binds preferentially to the poly(A)$^+$ tail of the RNA molecule. Poly(A)$^+$ RNA prepared from larch tissue could not be efficiently reverse-transcribed into cDNA using oligo-dT primed reactions. We, therefore, used random hexamer primers to construct our cDNA libraries. We do not know if the putative polysaccharide contaminant has any affect on the total yield of poly(A)$^+$ RNA. Neither do we know whether any reduction in the yield would be selective. Our assumption is that the binding of this contaminant is non-specific, but could affect mRNA species that are A-rich. However, it should do this regardless of whether the RNA came from juvenile or mature plants.

Sequences that are potential candidates for differential expression were identified using our hybridization scheme. However, all of the clones which were further examined were not differentially expressed in all tree families. These results demonstrate the effect of genetic background on gene expression and the importance at looking at multiple tree families. Whereas the data presented here showed that the variation in gene expression was due to genetic background, the reverse case could also occur. That is, differential gene expression associated with development could be masked by variation due to genetic background. Detection of these sequences would require working with one genotype.

As part of this work, we also identified clones for the ssu of Rubisco. These clones were obtained for two reasons. First, the cDNA libraries were constructed from RNA which was extracted from a variety of tissues, including foliage. We tested the integrity of the libraries by probing for known sequences. In addition, we have data which show that the level of chlorophyll is different between juvenile and mature trees (Hutchison et al. in prep.). Therefore, we wondered whether the genes involved with photosynthesis are differentially expressed.

The larch ssu-Rubisco showed strong conservation with the homologous sequence from other plants, both at the protein level and the nucleic acid level. The transcript produced was approximately 1000 nt in length, and was expressed in foliage, but not in roots. This is similar to the situation in other plants (Coruzzi et al. 1984). Finally, we saw no maturation-related expression of the ssu-Rubisco sequences. In a parallel series of experiments, we do have evidence that the chlorophyll *a/b*-binding protein is differentially expressed in juvenile and mature plants (Hutchison et al. in prep.).

Maturation is a largely unknown developmental process of great importance to the study and use of conifers. By understanding the underlying genetic mechanism(s) involved in maturation, we hope that this process can be manipulated for experimental and economic purposes. Our results to date demonstrate the complexity of the regulation of gene expression in conifers, and the difficulty of working with a heterogeneous genetic background. Nevertheless, we feel the molecular approach used in our study will ultimately succeed in identifying sequences that are differentially expressed between juvenile and mature plants.

Acknowledgments

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THE 25S, 18S, AND 5S RIBOSOMAL RNA GENES FROM *PINUS RADIATA* D.DON

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Introduction

Genes specifying the 18S, 25S, and 5S ribosomal RNAs (rRNAs) are present in thousands of copies in angiosperm genomes. The genes coding for the 18S and 25S rRNAs (rDNA) are linked and transcribed as a single unit, but are not linked to the genes coding for the 5S rRNA (5S DNA). Both sets of genes are organized in tandem arrays at one or a few chromosomal loci (for review, see Flavell 1986).

The length of the repeat unit for the rDNA varies between species from 7000 base pairs (bp) (e.g. soybean) to over 12 000 bp (e.g. wheat). This length variation is due to different amounts of intergenic DNA; that is, the DNA separating adjacent transcription units in the tandem arrays. The amount of intergenic DNA varies among plants of many species. Detailed studies in several species (e.g. pea, wheat) indicate that about half the intergenic DNA consists of tandem arrays of short repeats. In some instances the intraspecies variation in intergenic DNA length has been clearly correlated with the variation in the number of intergenic subrepeats.

The 5S RNA sequence is highly conserved across most classes of organisms; therefore, the coding region of the 5S DNA repeat unit is similarly conserved. The length of the repeat unit in angiosperms varies from 320 bp to 580 bp, with the coding region comprising about 120 bp. There is less information on genome organization for gymnosperms as compared to angiosperms. As a starting point the rRNA genes were characterized in *Pinus radiata* D. Don and compared to the organization of these genes in the angiosperms. The rDNA genes in *P. radiata* were cloned, localized on the chromosomes by *in situ* hybridization, and their genomic organization determined. The heterogeneity within and among individuals for these genes across a range of populations also investigated. The sequence for the 5S gene was deciphered and the transcribed region compared with comparable sequences in10 angiosperms.

Materials and Methods

Genomic DNA from suspension cultures of *P. radiata* was isolated by preparing protoplasts from cells and then extracting DNA from the protoplasts as described by Cullis (1979). The DNAs from 18 individual trees were isolated from disrupted pollen tubes. All DNAs were purified by banding on a cesium chloride/ethidium bromide gradient.

A fraction of the DNA enriched for rDNA and 5S DNA was isolated on a cesium chloride/actinomycin D gradient (Hemleben et al.1977). The rDNA and 5S DNA fractions were separated on a second, neutral, cesium chloride gradient without dye. In this second gradient the 5S genes banded at a lower density than the rDNA.

The enriched 5S DNA fraction was cloned into the *Bam*HI site of the vector pUC19. Clones containing the 5S gene were detected using the flax clone pBG13, which contains 10 tandem repeats of the flax 5S DNA repeat unit (Goldsbrough et al. 1981). One clone, designated 1627.39 and containing 5S homology, was subcloned into phages M13, mp18, and mp19, and into the plasmid pM13$^+$ of the Bluescript sequencing system (Stratagene Inc.). Both single strand and double strand dideoxy sequencing reactions (Sanger et al. 1977) were performed using BRL reagents and ^{35}S-dATP. The DNA sequences were analyzed using the computer program DNAsis.

Recombinant phage were plaque-purified from an unamplified library of partially digested Sau3A fragments of *P. radiata* DNA cloned in the vector EMBL4 (Frischauf et al. 1983). rDNA containing phage was selected using the flax rDNA clone pBG35 (Goldsbrough and Cullis 1981) as the heterologous probe.

The mapping of the genomic DNA was done using DNA extracted from the suspension culture cells. DNA was digested as recommended by the supplier (Boehringer Mannheim Gmbh) of the restriction enzymes, subjected to electrophoresis on agarose gels, and transferred to Nytran (Schleicher and Schuell). The probes were labelled by the oligolabelling method (Feinberg and Vogelstein 1983).

In situ hybridizations were done using the suspension culture cells as outlined by Gall and Pardue (1971). Briefly, air dried squash preparations were pretreated with RNAse A (100 µg/mL), washed in 2X SSC, dehydrated with enthanol, and air dried again. Tritiated probe (pBG35 or 1627.39, labelled to a specific activity of 10^6 to 10^7 cpm/µg by oligolabelling with equimolar amounts of [^{3}H]-dNTPs) was added to prepared cells in 2X SSC, 50% deionised formamide. The slides were incubated at 65°C for 10 min to denature both the chromosomal DNA and the probe. The slides were then incubated at 30°C for 16 h. Slides were washed for 5 min each, once in 2X SSC at 30°C, once in 1X SSC, 50% formamide at 30°C, and twice in 2X SSC at room temperature. Washes were followed by dehydration with ethanol, air drying, and coating with a 50% v/v dilution of Kodak NTB2 emulsion. The slides were stored at 4°C. Autoradiographs were developed at 15°C for 5 min in Dektol, fixed in Amfix, stained with Giemsa, and mounted for microscopy. Photographs were taken with Ilford PanF film using an Olympus photomicroscope with a x40 planapochromat objective.

Results

5S DNA

The plasmid library generated from the enriched pine 5S DNA fraction was screened with the heterologous probe from flax (pBG13). A clone 1627.39 was identified which contained a single *Eco*RI fragment of approximately the same size as the band observed when *Eco*RI digested genomic DNA was hybridized with the same probe.

The genomic organization of the 5S genes was determined by hybridizing partial *Bam*HI digests with the 1627.39 clone. An example of such an experiment is shown in Figure 1; a ladder of bands is produced in the partial digests. The bands on the ladder are spaced at equal increments in molecular weight, which indicates that genes are present as long tandem arrays. The repeat size of units on this ladder was calculated to be 525 bp and this corresponded exactly to the size of the insert in clone 1627.39.

Figure 1. Hybridization of clone 1627.39 to a genomic *Bam*HI partial digest. 2 µg DNA from cultured cells were digested with increasing enzyme concentrations (0 µ/µg to 20 µ/µg, left to right). Probing with ^{32}P-labelled 1627.39 generated a ladder-like pattern of bands, the repeat unit of which was 525 bp.

The sequencing of clone 1627.39 was accomplished using two systems. Firstly, the M13 bacteriophage system was used to produce single-stranded recombinants suitable for sequencing. Specifically, the entire *Eco*RI insert from the putative 5S clone was inserted into the *Eco*RI site for both mp18 and mp19. The two fragments produced from clone 1627.39 by digestion with *Eco*RI and *Bam*HI were directionally cloned into each vector. Secondly, the same cloning strategy was repeated using the plasmid pM13$^+$ of the Bluescript system (Stratagene). Double-stranded sequencing was conducted with recombinants from pM13$^+$. The putative 5′ to 3′ sequence is shown in Figure 2.

The transcribed region of the pine 5S gene was compared to that of flax and nine other angiosperms, using flax as a baseline (Figure 3). No additions or deletions were found; therefore, the coding region of the *P. radiata* 5S is 120 bp, as found previously for the angiosperms. Pairwise comparisons between flax and pine results in a homology value of approximately 89%. Homology values between 83% and 91% were obtained for comparisons of pine and the angiosperms listed. Identical comparisons between each of these angiosperms and flax gave homology values between 88% and 90%, except in two cases where a greater homology was observed. In the comparison between flax and pine, 13 base substitutions were apparent in the pine sequence. Three of these substitutions involved transversion mutations, while six of the substitutions occurred at unique sites as compared to all other angiosperms considered. Transitions account for the majority of the mutations observed in the comparison of pine 5S with the angiosperms.

CTTAAGTTTT GGGGGGACCG TGGGCGTTCA CGGAAAGGCC GCGCGACGCC ACTCCAAAAC

GCAGCGCCGT CGGTGGCCGC GGAACTTACG CCTTTTTTCT TCTCCCTCTC TCGTTTTCTT

TCTTCCTTTC CTTTTCCCCC TTCGTTCTTC CGCTGGTTTC GCCGGCTCCC CGCGGGGCGA

GACGCCGCAA GTCGGGGAGG AGGGGCGAGC TCCCGCGCCG AGCCGCTTCG GATCGGGGGC

CGTGGCTGCC CATGCCCTGC GCGTGGCTCG GCGGTCTCCC TTGAAGAGGG GGAGGGGCTG

AGATCTTTGC **GGGTGCGATC ATACCAGCGT TAATGCACCG GATCCCATCA GAACTCCGTA**

GTTAAGCGCG CTTGGGCTAG AGCAGTACTG GGATGGGTGA CCTCCCGGGA AGTCCTAGTG

TTGCACCCTC CCCCCCCTTT TTTGTGCGGC GCGCGGAGCC AGTGGAAGAT GACGCGAGGA

GAGTAGGTGA GGGGTTTAAA CCATATATAC TTTTGTAGGA ATTC

Figure 2. The 5′ to 3′ nucleotide sequence of the 5S RNA gene in pine. The transcribed region of the gene appears in bold type. The *Eco*RI site is shown at the 3′ end.

```
                  GT                            T      G  C      *
GGGTGCGATCATACCAGCACTAATGCACCGGATCCCATCAGAACTCCGAAGTTAAACGTG     a
  A      ACC T         A                               G         b
  A      ACC T                     ^                   G         c
A        ACC T                     ^             C     G         d
^        ACC T                     ^             C     G         e
-        ACC T                     ^             C     G         f
  A      ACC T         C           ^                   G         g
                                                       G         h
A   C                                           TC T   G   C     i
A   C                                            C               j


       T    C      GG             CC          T           T      *
CTTGGGCGAGAGTAGTACTAAGATGGGTGACCTCTTGGGAAGTCCTCGTGTTGCACCCCC     a
                    G             C                    T  T      b
                    G             C                    T  T^     c
                    G             C           T          TT^     d
                    G             C                      TTT     e
                    G          C  C                      T^^     f
                    G          C  C                      T^^     g
                    G             C                        T     h
      A             GC       G    C                      T T     i
  C                 G             C                      T       j

* Pine  a Flax  b Wheat  c Rye  d Broad bean  e Dwarf bean
f Sunflower  g Tomato  h Spinnach  i&j Yellow lupin
^ Indicates missing base
Only differences are shown
```

Figure 3. Comparison of the transcribed region of the 5S RNA gene of pine and 10 angiosperms. The sequences of flax and nine other angiosperms (Goldsbrough et al. 1982) were used to determine homology values for the 5S gene in pine. Values ranged from 83% to 91%.

Qualitative variation in 5S gene organization was estimated using a sample of 18 individuals chosen from across the *P. radiata* gene pool. DNA was collected from ruptured pollen tubes and digested with the restriction enzymes *Sac*I and *Hae*III. No differences were seen among any of the individuals on the blots of *Hae*III digests hybridized with clone 1627.39, but some differences were observed among individuals for the *Sac*I digest (Figure 4). However, it does not appear that there is any correlation between these bands and a particular group of individuals; that is, the variation among groups did not seem greater than that among individuals.

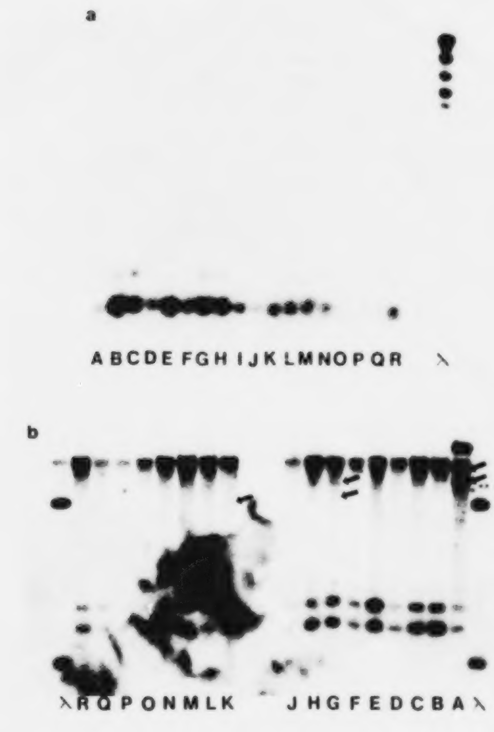

Figure 4. Clone 1627.39 hybridized to *Hae*III (a) and *Sst*I (b) restriction digests of individuals representing the *Pinus radiata* gene pool. The individuals sampled represent the following provenances: A-C Ano Neuvo; D-G Monterey; H-J Cambria; K-L Cedros; M-N Guadelupe; O-R Australia. Variant bands are marked with arrows.

The organization of the 5S genes in *P. radiata* was determined by *in situ* hybridization of chromosomes from rapidly dividing suspension cultured cells using clone 1627.39 as a probe. Hybridization of clone 1627.39 on many chromosomes indicated a wide dispersal of the 5S genes. This is in contrast to the pattern observed with wheat and maize, where a small number of specific sites for the 5S genes are observed (Appels et al. 1980, Mascia et al. 1981).

rDNA

The initial characterization of rDNA was made using flax rDNA as a heterologous probe. The hybridization of this probe to *P. radiata* genomic digests is shown in Figure 5a-d. The flax probe will only hybridize to coding regions of the rDNA repeat unit and any fragment which only contains intergenic DNA will not appear. The intergenic region was characterized using the rDNA-enriched fraction obtained from double gradient fractionation. When this fraction was

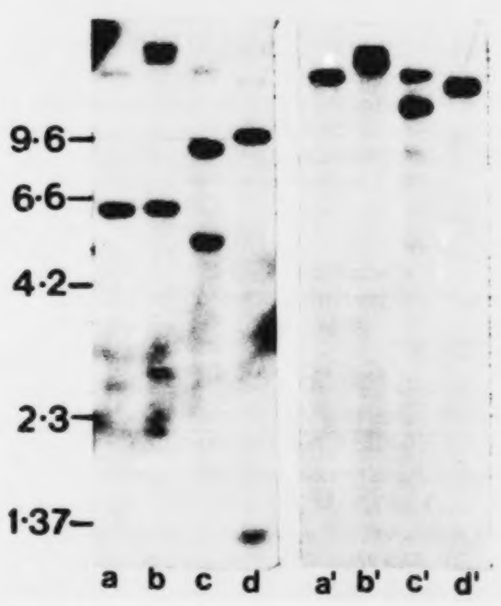

Figure 5. Hybridization of *P. radiata* DNA with labelled flax rDNA (a-d) and the enriched rDNA fraction (a'-d'). The DNA was digested with *Eco*RI (a,a'), *Bam*HI (b,b'), *Eco*RV (c,c'), and *Hind*III (d,d').

labelled and hybridized, the same basic pattern was generally observed as seen with the flax probe, but there were altered stoichiometries (Figure 5a′-d′). In the digests with *Eco*RI, *Bam*HI, and *Hind*III all bands observed with the enriched rDNA fraction were also seen with the flax probe. This means that all fragments containing intergenic DNA also contain some coding sequence. In the digestion with *Eco*RV (Figure 5 c,c′), a 12 kb band appeared in the hybridization with the enriched rDNA which was not labelled with the flax probe. Therefore, this band must only contain intergenic DNA.

A small number of bands can be seen when genomic DNA is digested with the four enzymes shown in Figure 5. However, this is not the case when the *Sac*I enzyme is used (Figure 8). It is not yet clear whether these multiple bands are due to the effects of methylation or to heterogeneity in the rDNA. Clearly, from the data in Figure 5, there cannot be a large amount of heterogeneity for the length of repeat unit, as this would be observed in multiple bands, especially in the case of the intergenic region delimited in the *Eco*RV digest.

The hybridization data can be used to construct a restriction map of rDNA (Figure 6). The repeat unit is approximately 27 kb, with little length heterogeneity. However, to accommodate all the fragments observed, the assumption has been made that the largest bands seen in the *Bam*HI, *Eco*RV, and *Hind*III digests are due to incomplete digestion of the DNA due to methylation or loss of restriction sites in the DNA. A number of rDNA-containing clones in the vector EMBL4 have been isolated. A preliminary restriction map of these clones has confirmed the overall pattern of the map shown in Figure 6. However, there are a number of small fragments which have not been observed in genomic blots present in the phage clones. These may be caused by rearrangements which have occurred in the cloning procedures or be due to heterogeneity in the rDNA. A larger number of phage clones will have to be mapped before this matter is settled.

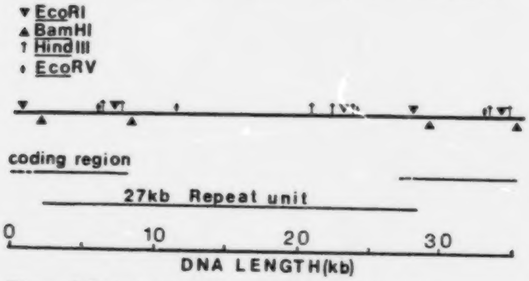

Figure 6. Restriction map of the DNA repeat unit.

In situ hybridization techniques showed a number of sites of rDNA on the chromosomes (Figure 7). The multiple sites can be seen in both mitotic figures as well as in the interphase nuclei. From the data presently available, it would appear that there are at least 10 major sites of rDNA, as well as a number of minor sites. In this regard the conifer genome would appear atypical among higher plant genomes.

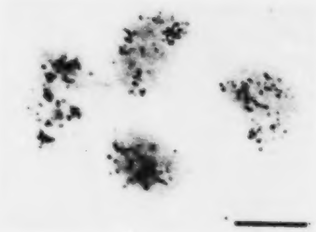

Figure 7. *In situ* hybridization of ^{3}H-labelled plasmid pBG35 to *P. radiata* chromosomes and interphase nuclei. The bar represents 20 μm.

There is both quantitative and qualitative variation in rDNA among the *P. radiata* gene pool. The rDNA amount can vary more than twenty-fold (Cullis et al. unpublished). Restriction fragment length polymorphisms can also be observed for rDNA, especially by using the restriction enzyme *Sac*I (Figure 8).

Discussion

Organization of rDNA and 5S DNA in *P. radiata* has similarities to the known characteristics for angiosperms. Both sets of sequences occur in tandem arrays, as has been shown for all angiosperms characterized to date. The repeat unit for pine 5S genes is 524 bp, which is within the range observed for angiosperms. The rDNA repeat unit in *P. radiata*, at 27 kb, is more than twice as long as the longest angiosperm rDNA so far characterized. In all cases of angiosperms with long rDNA repeat units, a substantial part of the length is made up of a repeating element in the intergenic region. There is also a large

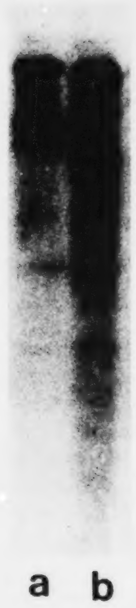

Figure 8. Hybridization with the enriched rDNA fraction of *SacI* digested DNA from two individual trees.

amount of length heterogeneity associated with such subrepeats. Although the intergenic region appears to contain a highly repeated sequence, there are no obvious polymorphisms associated with this repeat unit. Both 5S DNA and rDNA appear to be located at a large number of sites within the genome. This is unusual because, for all angiosperms so far studied, the rDNA and 5S DNA occur at only a few chromosome sites.

The coding region of the 5S repeat unit is homologous with the 10 angiosperm species with which it was compared at a level of 83% or higher. This clearly demonstrates the conservation of this sequence between angiosperms and gymnosperms.

For both 5S DNA and rDNA there were quantitative and qualitative polymorphisms when individuals across the gene pool were sampled. Overall, there appeared to be no more variation among provenances than observed among individuals derived from the same provenance. Whether or not this observation holds true for the genome as a whole awaits further information on the variation in other sets of sequences.

Acknowledgments

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CLONING AND CHARACTERIZATION OF A HIGHLY CONSERVED, PINACEAE-SPECIFIC CHLOROPLAST DNA COMPONENT FROM SCOTS PINE (*PINUS SYLVESTRIS* L.)

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Abstract

The genetic structure of Scots pine *Pinus sylvestris* L. was studied by cloning a prominent *Eco*RI fragment 1600 base pairs long, from a total DNA preparation. The cloned fragment hybridized specifically to restriction endonuclease fragments in a purified chloroplast DNA preparation from Scots pine. Southern blot experiments showed that the cloned fragment displayed little or no restriction fragment length, or sequence heterogeneity, when tested against 11 other pines and several other members of Pinaceae. At hybridization stringencies about 95% the cloned fragment hybridized only to members of the Pinaceae when tested among both angiosperms and gymnosperms. At hybridization stringencies of 50%, the cloned fragment hybridized to other plants. In contrast, the conserved chloroplast gene *psb*A and the nuclear gene for 18S rRNA manifest restriction fragment length heterogeneity, as well as homology to DNA, from all families of plants tested at high levels of stringency. We conclude that the cloned fragment represents a highly conserved, Pinaceae-specific chloroplast DNA segment.

Introduction

Conifers represent a phylogenetically well separated class of plants. Pines, being more than 100 million years old, show an advanced evolutionary age and a pronounced genetic conservatism (Mirov 1967). Does this mean that the genetic structure of pines is more highly conserved compared to other plants? Conifers also differ physiologically from angiosperms in a number of ways. For example, unlike angiosperms the conifers are capable of forming substantial amounts of chlorophyll in the absence of light (Castelfranco 1983). The chloroplasts of dark-grown pine tissue are well-developed and contain both chlorophyll *a* and *b* (Lewandowska and Öquist 1980).

About half of the genetic information needed for the photosynthetic apparatus is located in the chloroplast genome (reviewed by Palmer 1985). The chloroplast genome is well described for a number of angiosperms, and some mosses and algae (Palmer 1985, Rochaix 1985). The complete DNA sequences of chloroplast genomes from *Marchantia polymorpha* and *Nicotiana tabacum* were recently reported (Ohyama et al. 1986, Shinozaki et al. 1986). Gene structure and gross gene content are well conserved in the chloroplast genomes studied (Palmer and Stein 1987). Pines have been found to have a chloroplast genome similar in size to that of other plants (Kondo et al. 1986, Szmidt et al. 1986, White 1986), but more variability has been observed in gene orders and genome size (Palmer 1985, Rochaix 1985).

The chloroplast genome is normally composed of two inverted repeat structures, separating a large single copy region from a small single copy region. In pines, however, it seems as if one of the inverted repeats is lacking (G. Howe and S. Strauss, pers. comm., J. Lindholm and A. Szmidt, pers. comm.). The distinct differences between the chloroplast genome of pines and other plants, combined with its importance for the photosynthetic apparatus, prompted us to clone and study genetic components from the chloroplast of Scots pine (*Pinus sylvestris* L.).

In this paper we present the cloning and characterization of a highly conserved, Pinaceae-specific chloroplast DNA component from Scots pine.

Materials and methods

The plant material was taken from the Faculty of Forestry, Swedish Agricultural University, Umeå. Species used are listed below. Pinaceae: *Pinus sylvestris* L., *P. contorta* Dougl., *P. cembra* L., *P. resinosa* Ait., *P. ponderosa* Dougl., *P. canariensis* C. Smith, *P. flexilis* James, *P. halepensis* Mill., *P. pinea* L., *P. taeda* L., *Larix sibirica* Ledeb., *Picea abies* (L.) Karst., *Abies halophylla*, *Pseudotsuga menziesii* (Mirb.) Franco. Cupressaceae: *Cupressus sempervirens* L., *Juniperus communis* L., *Calocetrus decurrens*, *Mikrobiota orientalis*. Taxodiaceae: *Sequoia sempervirens* (D. Don) Endl. (reduced), *Sequoiadendron giganteum* (Ldl.) Buchh., *Athrotaxis cypressoides*, *Taxus cuspidata* Sieb. and Zucc. Other plants: *Pisum sativum* L., *Araucaria angustifolia*, *Podocarpus neriifolia*, *Ephedra gerardiana*, *Nicotiana tabacum*.

The bacterium *Escherichia coli* strain C600 and plasmid pUC8 were used for cloning purposes (Vieira and Messing 1982). The *psb*A specific probe used was an internal 769 bp *Pst*I/*Xba*I fragment from the spinach clone pPSII32/1, kindly provided by R.G. Herrmann. The 18S rDNA specific probe used was an *Eco*RI fragment (Ver 6) from *Vicia faba*, kindly provided by K. Yakura (Yakura et al. 1984).

DNA was prepared according to Murray and Thompson (1980). Chloroplast DNA was prepared according to Szmidt et al. (1986).

Preparation of plasmid DNA and cloning techniques was according to Maniatis et al. (1982). Restriction endonocleases, ligase, and other enzymes were used according to manufacturer's specifications.

The probes for hybridization were labelled with ^{32}P by Amersham's Multi-prime labelling system to a specific activity exceeding 10^9 cpm/µg DNA. Only purified fragments, containing no plasmid vector DNA, were used as probes for Southern (1975) hybridizations. The following conditions were used for high level stringency hybridization: hybridization 6XSSC at 65°C, overnight washing, 0.1XSSC at 65°C, 30 min or as described in the figure legends. Conditions for low level stringency hybridization were: hybridization, 6XSSC at 65°C, overnight washing, 0.1XSSC at 42°C, 45 min or as described in the figure legends. Conditions for medium level stringency hybridizations were: hybridization, 6XSSC at 65°C, overnight washing, 0.1XSSC at 55°C, 30 min.

Results

Cloning of repetitive DNA from *Pinus sylvestris*

DNA was isolated from *P. sylvestris* as described in Materials and Methods. The isolated DNA was cut with *Eco*RI and analyzed on agarose gels. Several prominent bands could be seen (data not shown). A strong band, 1600 bp in size, was cut out of the gel, purified, and cloned into the plasmid pUC8. The cloned DNA fragment was analyzed for the existence of additional restriction endonuclease sites. The fragment contained two *Bam*HI sites, one *Cla*I site, but no site for *Sal*I. The order of restriction sites was *Eco*RI-200 bp/*Bam*HI-350 bp/*Cla*I-50 bp/*Bam*HI-1000 bp/*Eco*RI.

The DNA preparation from which the 1600 bp *Eco*RI fragment was cloned contained both nuclear and organelle DNA. The existence of several prominent DNA bands in the endonuclease restricted DNA sample indicated either the existence of nuclear repetitive DNA components and/or a high contamination of organelle DNA in the total DNA preparation. To verify the origin of the cloned 1600 bp DNA fragment, we performed a Southern hybridization between this fragment and a purified chloroplast DNA preparation from *P. sylvestris*. The chloroplast DNA was cut with restriction endonucleases *Eco*RI, *Sal*I, and *Bam*HI. The result (Figure 1) shows that the cloned fragment hybridizes strongly with the chloroplast DNA preparation. An analysis of the restriction fragment pattern of the chloroplast DNA from the agarose gels revealed that the cloned fragment hybridized to bands clearly visible as single bands in all three lanes. The size of the *Eco*RI fragment also matches the 1600 bp size of the cloned fragment. In the *Bam*HI digestion, two bands can be seen which fit the map constructed over the cloned fragment. The fragment does not contain any site for *Sal*I, and this also can be seen from the Southern blot in Figure 1. We conclude that the cloned 1600 bp fragment is a component of the chloroplast genome of Scots pine.

To understand the extent to which this fragment was conserved, DNA was purified from several

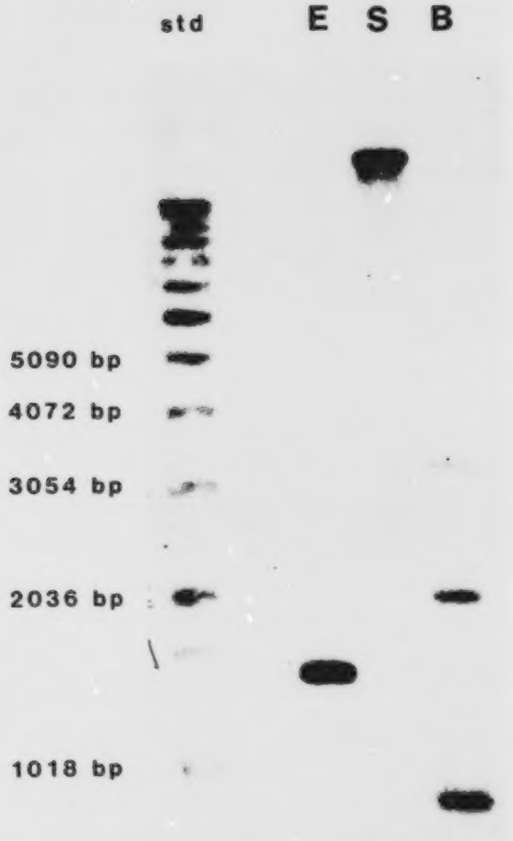

Figure 1. Southern blot of the 1600 bp *Eco*RI fragment and purified chloroplast DNA from *P. sylvestris*. The following restriction endonucleases were used: *Eco*RI (lane E), *Sac*I (lane S), and *Bam*HI (lane B). Molecular weight standard is indicated on the left.

Pinaceae species and cut with several enzymes. Under conditions of high stringency, Figure 2 shows that the 1600 bp *Eco*RI fragment hybridized with a 1600 bp fragment for the five pine species and *L. sibirica* in the *Eco*RI digest. Small variations in the length of the fragment were seen. The fragments from *P. cembra* and *P. resinosa* Ait. were slightly smaller and larger, respectively, than those of other conifers. Also, when using restriction enzymes *Msp*I and *Rsa*I, which recognize a four base sequence, conservation was remarkably high. In all 11 pines tested (except *P. cembra*), and in *L. sibirica*, the 1600 bp fragment hybridizes to a single band of identical size, about 2000 bp and 1000 bp for the two enzymes *Msp*I and *Rsa*I, respectively. As high stringency was used, the DNA sequence homology between the 1600 bp fragment and its counterpart from different members of the Pinaceae tested must, therefore, be higher than 95%. As a control, we used DNA from *Pisum sativum* L. in the hybridizations (Figures 1A and B, lane 7). No positive hybridization could be detected for the given hybridization conditions.

It is apparent that the cloned 1600 bp fragment is highly conserved within the pine family, both with respect to restriction endonuclease pattern and DNA sequence. In addition, the finding that the fragment did not hybridize to pea at the level of stringency used prompted us to investigate if it could also be Pinaceae-specific.

DNA was isolated from a number of plants, including tree species which are considered to be closely related to the Pinaceae, belonging to the Cypressaceae and the Taxodiaceae. The isolated DNA was cut with *Hind*III and run on agarose gels. The DNA was subjected to Southern hybridizations using the 1600 bp *P. sylvestris* fragment as a probe. Two levels of stringency were used, representing 95% and 50% homology at the DNA level.

Results of the hybridization of the 1600 bp fragment to different gymnosperm families is shown in Figures 3A (high stringency) and 3B (low stringency). No hybridization can be detected to any plant outside the Pinaceae using a high level of stringency. This result again confirms that the 1600 bp fragment is highly conserved within the Pinaceae. When using a lower level of stringency, however, hybridization to other families can be detected. The hybridization was stronger to the Taxodiaceae and to angiosperms than to the Cupressaceae. Fragment sizes also differ in species outside the Pinaceae. Some conservation can be seen among the Taxodiaceae. We conclude that the isolated 1600 bp fragment can be used as a specific probe for the Pinaceae.

Hybridization of a chloroplast *psb*A gene and a plant nuclear 18S rRNA gene to plant DNA

The same filters used in the experiments described above (Figure 3) were reprobed at a medium level of stringency with radioactively-labelled *psb*A and 18S rDNA probes. The result

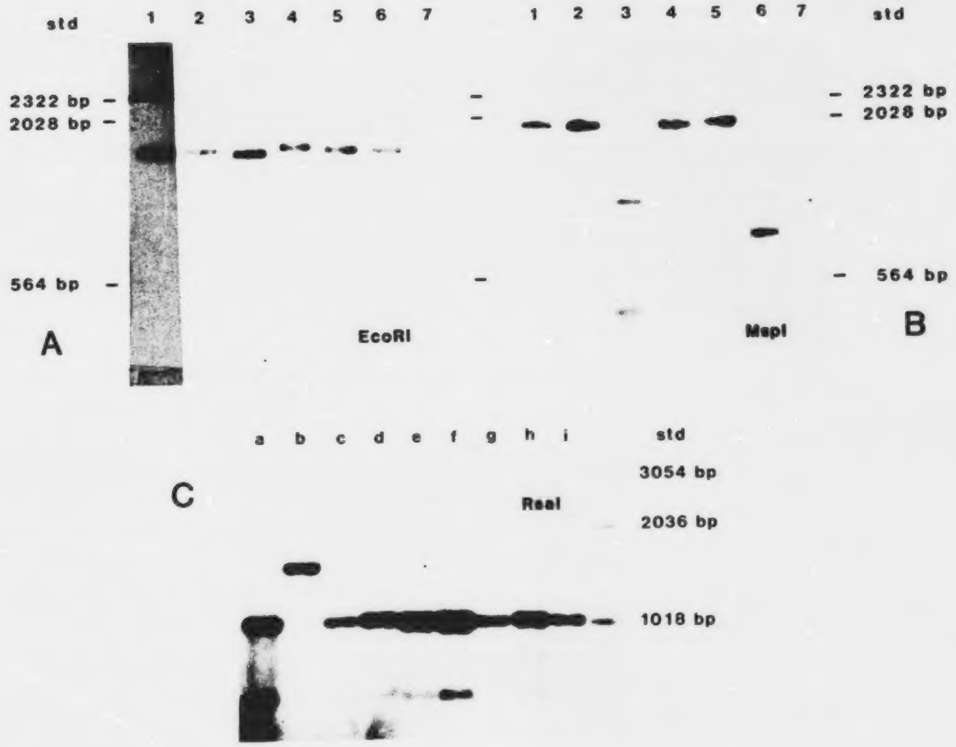

Figure 2. Southern blot between cloned 1600 bp *Eco*RI fragment and pine DNA from several spp.

Panel A: *Eco*RI-digest, 1, *Pinus sylvestris;* 2, *P. contorta* 727; 3, *P. cembra;* 4, *P. resinosa;* 5, *P. ponderosa;* 6, *Larix sibirica;* 7, *Pisum sativum.* Hybridization conditions; 60°C and 6XSSC. Washing conditions; 66°C and 0.1XSSC, 90 min.

Panel B: *Msp*I-digest, same as A. Hybridization conditions; 60°C and 6XSSC. Washing conditions; 60°C and 0.1XSSC, 45 min.

Panel C: a, *Pinus canariensis;* b, *P. cembra;* c, *P. contorta;* d, *P. flexilis;* e, *P. halepensis;* f, *P. pinea;* g, *P. ponderosa;* h, *P. sylvestris;* g, *P. taeda.* Hybridization conditions; 65°C and 6XSSC, o.n. Washing conditions; 62°C and 0.1XSSC, 45 min.

is shown in Figure 4. At higher levels of stringency, both probes hybridized, but only weakly, to several plant DNA samples.

Despite the fact that the *psb*A gene is highly conserved, a variation in restriction length was observed among the plants tested (Figure 4, left panel). Within the different subclasses tested, however, similarities were seen. *P. sylvestris* and *L. sibirica* carries *psb*A on a fragment of the same size (about 1900 bp). Also, in *Picea abies, Abies halophylla,* and *Pseudotsuga menziesii* we observed *psb*A hybridization to a 3100 bp fragment. The same similarity in band length can be observed between the Cupressaceae and the Taxodiaceae. It is also interesting to note that the *psb*A probe hybridizes to two large restriction fragments in *A. halophylla, Pseudotsuga menziesii,* and in all species tested belonging to the Taxodiaceae. This result is presumably due to the existence of a conserved *Hind*III site in the middle of the *psb*A gene.

When the 18S rDNA probe was used, a larger restriction fragment length heterogeneity was observed, especially among the Pinaceae. The

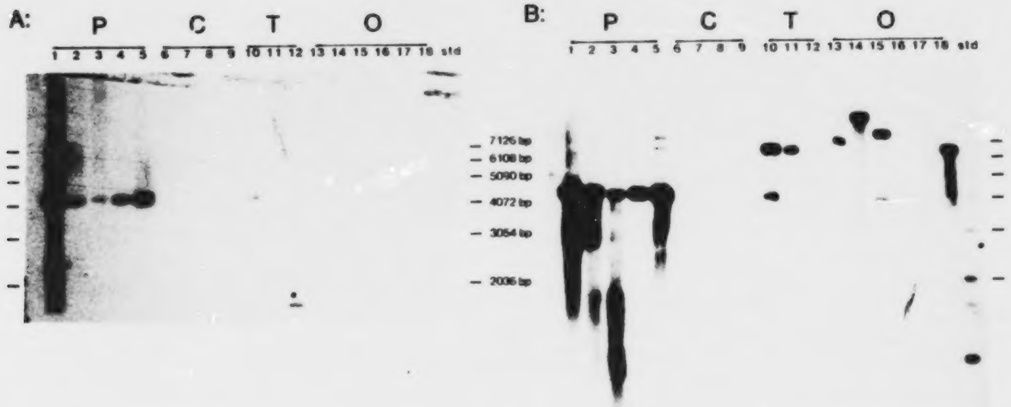

Figure 3. Southern blot between cloned 1600 bp *Eco*RI fragment and DNA cut with *Hind*III from different plants.

The following plants were used as DNA sources: P, Pinaceae; C, Cupressaceae; T, Taxodiaceae; O, Other plants: 1, *Pinus sylvestris*; 2, *Larix sibirica*; 3, *Picea abies*; 4, *Abies halophylla*; 5, *Pseudotsuga menziesii*; 6, *Cypressus sempervirens*; 7, *Juniperus communis*; 8, *Calocetrus decurrens*; 9, *Mikrobiota orientalis*; 10, *Sequoia sempervirens* (reduced); 11, *Sequoiadendron gigantum*; 12, *Athrotaxis cypressoides*; 13, *Araucaria angustifolia*; 14, *Podocarpus neriifolia*; 15, *Taxus cuspidata*; 16, *Ephedra gerardiana*; 17, *Pisum sativum*; 18, *Nicotiana tabacum*.

Panel A: Hybridization conditions (high stringency); 65°C and 6XSSC, o.n. Washing conditions; 65°C and 0.1XSSC, 30 min.

Panel B: Hybridization conditions (low stringency); 65°C and 6XSSC, o.n. Washing conditions; 42°C, 45 min.

large fragment sizes seen in many of the other species makes it difficult to interpret this variation. The large fragment size is probably due to large spacer regions in the rDNA cluster that do not contain any sites for *Hind*III (see Cullis, these proceedings).

Discussion

We have described the cloning and isolation of a 1600 bp *Eco*RI DNA fragment from *P. sylvestris* that is specific for members of the Pinaceae and, in addition, is highly conserved within that family. The 1600 bp *Eco*RI fragment was isolated from a total DNA preparation of *P. sylvestris*. We conclude, however, that the cloned DNA fragment is a chloroplast DNA component because of the specific hybridization obtained to purified chloroplast DNA from *P. sylvestris*.

Conifers, pines in particular, show an advanced evolutionary age and a pronounced genetic conservatism. Therefore, it is perhaps not surprising that DNA sequences exist that show a high degree of homogeneity and conservation in the Pinaceae. It is, however, interesting to observe that the 1600 bp fragment showed a higher degree of conservation than the photosynthesis gene *psb*A.

The finding that the 1600 bp fragment was both well conserved and highly specific for the Pinaceae prompted us to investigate if the same was true for other genetic markers that could be considered as being conserved through evolution. We chose the chloroplast gene *psb*A, coding for the reaction center polypeptide D1 in photosystem II, and a part of the nuclear 18S rRNA gene. Both genes are considered to be well conserved through evolution, especially the *psb*A gene, where the complex structure of the reaction centre imposes many constraints on the

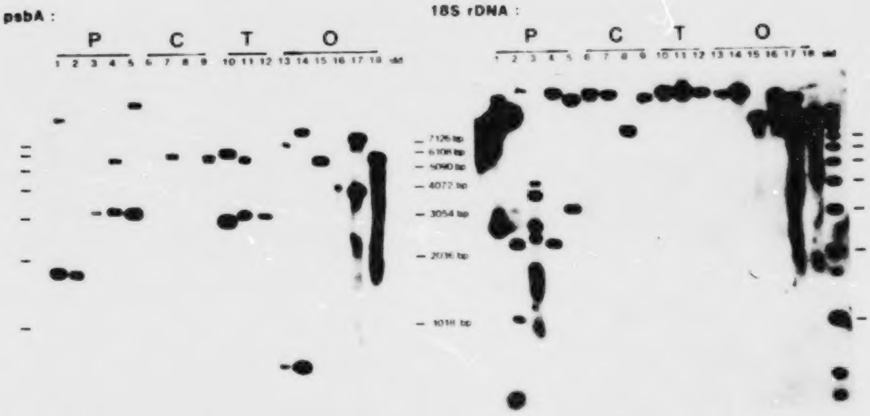
psbA :
P
C
T
O
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 std
18S rDNA :
P
C
T
O
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 std
7126 bp
6108 bp
5090 bp
4072 bp
3054 bp
2036 bp
1018 bp

Figure 4. Southern blot of DNA, from different plants, cut with *Hind*III using a chloroplast *psb*A gene and nuclear 18S rDNA as probes. The following plants were used as DNA sources: P, Pinaceae; C, Cupressaceae; T, Taxodiaceae; O, Other plants: 1, *Pinus sylvestris*; 2, *Larix sibirica*; 3, *Picea abies*; 4, *Abies halophylla*; 5, *Pseudotsuga menziesii*; 6, *Cypressus sempervirens*; 7, *Juniperus communis*; 8, *Calocetrus decurrens*; 9, *Mikrobiota orientalis*; 10, *Sequoia sempervirens* (reduced); 11, *Sequoiadendron gigantum*; 12, *Athrotaxis cypressoides*; 13, *Araucaria angustifolia*; 14, *Podocarpus neriifolia*; 15, *Taxus cuspidata*; 16, *Ephedra gerardiana*; 17, *Pisum sativum*; 18, *Nicotiana tabacum*.

Panel *psb*A: Hybridization conditions (medium stringency); 65°C and 6XSSC, o.n. Washing conditions; 55°C and 0.1XSSC, 30 min. Probe: *psb*A fragment from pea.

Panel 18S rDNA: Hybridization conditions (medium stringency); 65°C and 6XSSC, o.n. Washing conditions; 55°C and 0.1XSSC, 30 min. Probe: VER6 containing the 18S rDNA gene from *Vicia faba*.

amino acid sequence. The same can be argued for components of the translational apparatus of 18S rRNA.

We wonder whether the 1600 bp fragment contains genetic information for biological activity. Unfortunately too little is known at the present stage about the genetic structure of pine chloroplasts to be able to assign any genes or other genetic function to the fragment identified by the 1600 bp probe in the Southern hybridization to Scots pine chloroplast DNA. We partially sequenced the 1600 bp fragment. The DNA sequence obtained so far shows a homology to the sequenced chloroplast DNA of *Nicotiana tabacum*. However, homologous regions have been found at several positions on the tobacco chloroplast and a conclusive answer to which region the 1600 bp fragment shows the strongest homology has to await complete sequence of the fragment.

A pine-specific repetitive DNA component has recently been cloned and partially sequenced (R. Sederoff, pers. comm.). The partial DNA sequence obtained from the 1600 bp fragment of Scots pine shares no apparent homology with the repetitive DNA.

DNA sequences specific for biological species have been obtained before, especially among bacteria. A recent example is a 153 bp Sau3A fragment highly specific for the forest form of *Onchocerca volvulus* (Erttman et al. 1987). Species-specific DNA fragments can be thought to carry biological information distinguishing species at the phenotypic level. It is speculative if the 1600 bp fragment carries information that is phenotypically important for members of Pinaceae and less important for other plants, including other conifers.

Acknowledgments

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IN VIVO TRANSFORMATION OF HYBRID POPLARS: CLONAL VARIATION - A PRELIMINARY INVESTIGATION

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Abstract

As a natural host of *Agrobacterium tumefaciens*, *Populus* is a model system for transferring foreign genes to woody perennials by Ti plasmid-mediated transformation. Here we report clonal variation in susceptibility to *Agrobacterium tumefaciens* strain A208 for four *Populus deltoides* x *nigra* clones and one clone of *Populus nigra* x *maximowiczii*, as demonstrated by gall formation, phytohormone-independent growth *in vitro*, and opine production.

Introduction

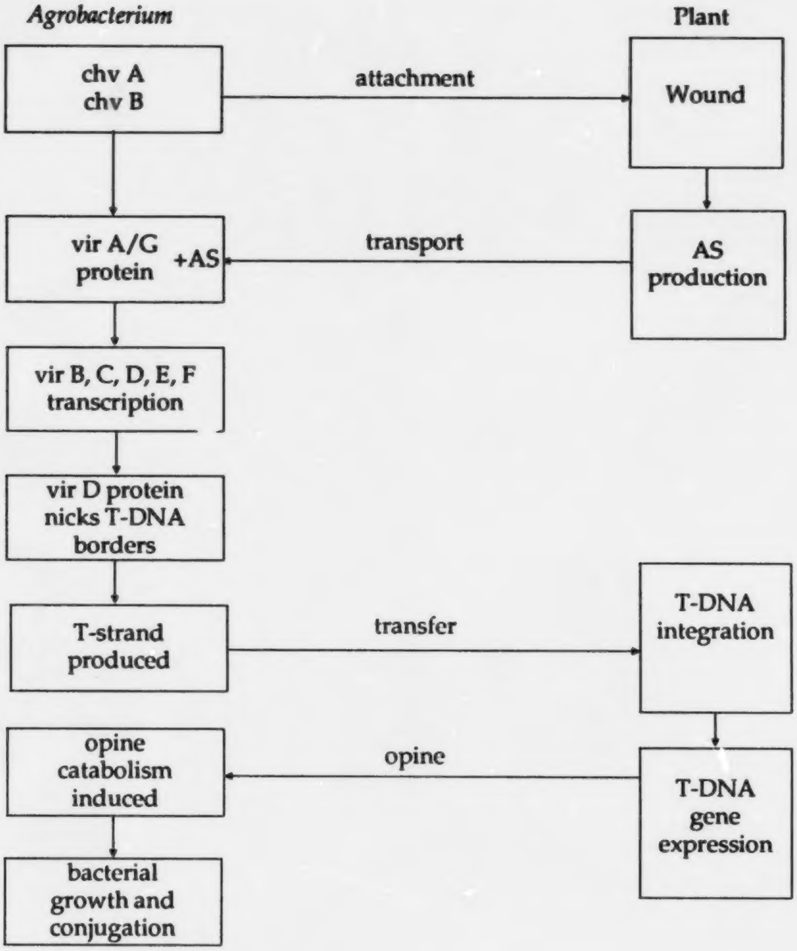
Application of genetic engineering techniques to introduce economically important genetic traits into forest trees offers a potential for rapid genetic improvement as compared to conventional breeding programs. *Populus* (poplar), a fast growing woody perennial, is economically important as a source of raw material for the pulp and paper industry. The genus is a natural host for *Agrobacterium tumefaciens*, which causes crown gall disease. This characteristic makes *Populus* spp. amenable to one of the most effective methods of altering the DNA content of tree genomes; we can manipulate the natural transformation of this organism to achieve improved clones. *A. tumefaciens* strains harbouring Ti (tumor-inducing) plasmids incite neoplastic hyperplasias in the presence of wounded plant tissue. These galls affect most dicotyledons and some monocotyledons and appear primarily at the crown separating stem from roots. Although bacterial cells are necessary for the infection process they are not necessary for continued growth and maintenance of the tumorous condition. The susceptibility of *Populus* hybrids to natural crown gall infections is illustrated by a severe outbreak of the disease in a nursery in France (Nesme et al. 1987) where more than 120 different *A. tumefaciens* isolates were implicated. In addition, stands of poplar hybrids have exhibited aerial galls up to 30 cm in diameter on trunks and branches, and from these a pathogenic *A. tumefaciens* strain has been isolated (Dochinger 1969).

The host ranges of *A. tumefaciens* strains are determined primarily by the Ti plasmid itself (Loper and Kado 1979, Thomashow et al. 1980), although the chromosomally-borne attachment genes also play a role (Matthysse 1987). The most important gene appears to be that coding for cytokinin synthesis in the T-DNA. A primary role is played by the host plant, because levels of susceptibility vary within plant species and cultivars (Holbrook and Miki 1985, Owens and Cress 1985). A summary of *A. tumefaciens*/plant interactions necessary for successful infection is depicted in Figure 1.

Materials and Methods

Bacterial Strain

Agrobacterium tumefaciens strain A208 (pTiT37) is an oncogenic, nopaline-utilizing transconjugate of strains T37 and A136 (a plasmidless derivative of C58) which confers resistance to rifampicin (5 µg/mL) and naladixic acid (50 µg/mL) (Sciaky et al. 1978). *A. tumefaciens* was maintained as streak cultures on solid medium M523 (Kado et al. 1972) and as glycerol stocks. For gall induction, a single colony was used to inoculate 10 mL of AB minimal medium (Watson et al. 1975) and incubated for 18 h at 29°C with orbital



Agrobacterium
Plant
chv A
chv B
attachment
Wound
vir A/G
protein
+AS
transport
AS
production
vir B, C, D, E, F
transcription
vir D protein
nicks T-DNA
borders
T-strand
produced
transfer
T-DNA
integration
opine
catabolism
induced
opine
T-DNA
gene
expression
bacterial
growth and
conjugation

Figure 1. *Agrobacterium*/Plant Interactions

shaking (240 rpm). Absorbance of the culture at 550 nm indicated a bacterial concentration of 1 x 10^8 organisms/mL. The bacterial cells were pelletted, washed twice with MS salts of pH 5.8 (Murashige and Skoog 1962), and resuspended in an equal volume of MS salts.

Plant Material

Five hybrid poplar clones, initially selected for varying degrees of field susceptibility to the herbicide simazine, were obtained as cuttings from the Ministry of Natural Resources, Brockville, Ontario. The characteristics of each hybrid are detailed in Table 1.

To provide shoots for inoculation, dormant stem cuttings from each parental clone were placed in 12 cm pots of 2:1:1 (soil:peat:perlite) plus 650 gm/cubic yard 14-14-14 slow-release fertilizer. These cuttings were maintained in the greenhouse at 10-32°C, 16 h day/8 h night. Natural daylight was supplemented with cool white fluorescent lighting.

When shoot length reached approximately 7-10 cm, galls were induced by wounding the first or second apical internode and the lowest green stem segment of the shoot. Wounding was immediately followed by inoculation with 10 μL A208, prepared as described. Subsequently, the

Table 1. Hybrid poplar clones for simizine resistance research

Clone number	Parent cross	Origin	Simazine tolerance
DN74	*P. deltoides* (D 35) x *nigra* (N 3)	Maple, Ontario	High
DN70	*P. deltoides* (D 35) x *nigra* (N 3)	Maple, Ontario	Medium
DN106	*P. deltoides* (D 36) x *nigra* (N1 + 2 mixed)	Maple, Ontario	High
DN112	*P. deltoides* (D 32) x *nigra* (N1 + 2 mixed)	Maple, Ontario	Low
NM6	*P. nigra* x *maximowiczii* (MAX - 5)	Munden, W. Germany	High
NM1	*P. nigra* x *maximowiczii*	Tokyo, Japan	Low

wounded area was completely wrapped with ethanol-disinfected parafilm strips for a period of 72-96 hours. This maintained a crucial microenvironment of high humidity at the inoculation site during transformation for these species. After unwrapping the wound site, plants were maintained in the greenhouse with periodic pruning. Control plants were wounded and inoculated with 10 µL sterile MS salts medium, then wrapped and maintained in the same manner as the test plants. For each poplar hybrid clone all shoots of each of five cuttings were inoculated apically and/or on the lower greenstem segment. Shoots of one stem cutting were treated as controls.

In vitro Culture of Galls

To establish cultures of gall tissue, gall material was excised from the stem and surface-sterilized with a 20 min wash in 2% (v/v) sodium hypochlorite, 0.1% v/v Tween 20, and 0.2% (w/v) Sparkleen, followed by a 5 min treatment in 5% formalin. The tissue was rinsed several times in sterile distilled water. The surface of the gall was peeled, and the core sliced and chopped into segments. These segments were cultured on Murashige and Skoog medium containing 3% sucrose, 500 µg/mL carbenicillin, and no growth regulators. The tissue was cultured at 25°C under 16 h day/8 h night (2500 lux). Callus tissue was transferred at monthly intervals. Shoots that developed spontaneously were removed and placed in separate dishes containing the same medium. After further elongation the shoots were transferred to 1.5 L Mason jars and maintained with monthly transfers to fresh medium.

Opine Analysis

Between 20 and 200 mg fresh tumor callus and non-tumor callus were weighed and ground in a volume of extraction buffer (1 µL buffer per mg tissue). The extraction buffer contained, per mL: 0.1 mL 1M Tris pH 8.0, 0.1 mL 1% ascorbic acid pH 8.0, 0.1 mL 1% cysteine, 0.335 mL 1.5M sucrose, and 0.365 mL water. Tubes were spun 5 min at 8000 rpm in a microcentrifuge. A volume (10-30 µL) of the supernatant was mixed with an equal volume of incubation solution. The remaining supernatant was used as a zero-time control. The incubation mixture for nopaline detection contained: 20 mg NADH, 66.6 µL 1M $NaPO_4$ pH 6.8, 83 µL 240 mM α-ketoglutarate, 83 µL 240 mM arginine, and 50 µL H_2O. Incubation was carried out for 2 h at 25°C. Electrophoresis was carried out in a running buffer of 5% formic acid, and 15% acetic acid at a current of 17-24 milliamps and 400V for 60 min. Nopaline control standards were spotted at both sides of test samples. When electrophoresis was complete the paper was dried, sprayed with phenanthrenequinone solution, and observed under uv light. Nopaline standard was purchased from Sigma Chemicals.

Results

The first response to inoculation with phytopathogenic *A. tumefaciens* strain A208 occurred at the wound site within two weeks. There was a proliferation of cells and swelling in the wounded area. Tannin-like substances are also produced at wound sites and these initial responses were observed on both inoculated and uninoculated control plants. However, responses terminated on the control plants at three weeks and no further growth at the wound site was evident. At successfully infected sites, tumors were allowed to develop for four months prior to excision. Tumors were of varying sizes, typically spherical, with rough surfaces; some exhibited shoot buds.

Tumors developed at apical wound sites on all clones more frequently than at the lower greenstem wound sites (Figure 2). The percentage of wounds that developed tumors from *in vivo* infection for each clone varied from 33% to 0% (Figure 3). Variation in tumor formation for apical versus woody inoculations among clones was consistent. On average, approximately 35% more tumors were obtained on apical versus woody inoculations. There was, however, evidence of clonal interaction for this trait (Figure 4). Clones of the various *Populus* hybrids varied considerably with respect to tumor formation response. The *P. deltoides* x *nigra* clones all exhibited some degree of tumor formation, where *P. nigra* x *maximowiczii* was apparently resistant to tumor formation both apically and in the greenstem location.

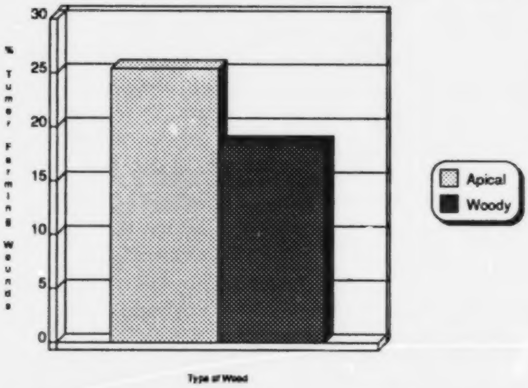


Figure 2. A208 (pTiT37 nopaline) tumor formation - Wound response means

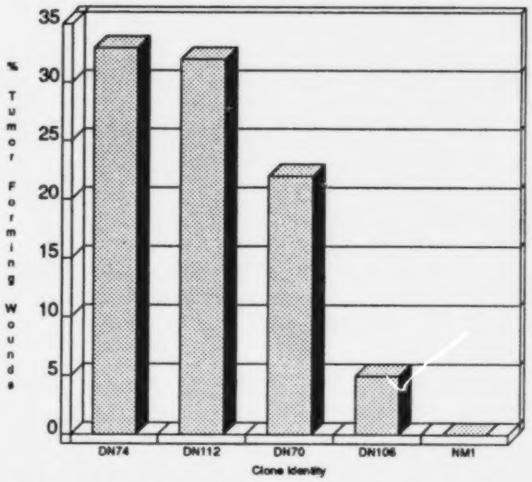


Figure 3. A208 (pTiT37 nopaline) tumor formation - Clonal means

Opine analysis of culture tumor callus showed phenanthrenequinone-positive spots that comigrated with the nopaline standard. Untransformed poplar callus showed no spots that comigrated with nopaline. Once in culture various samples of the tumor tissue spontaneously developed abnormal shoots and/or roots that differentiated and grew in the absence of phytohormones.

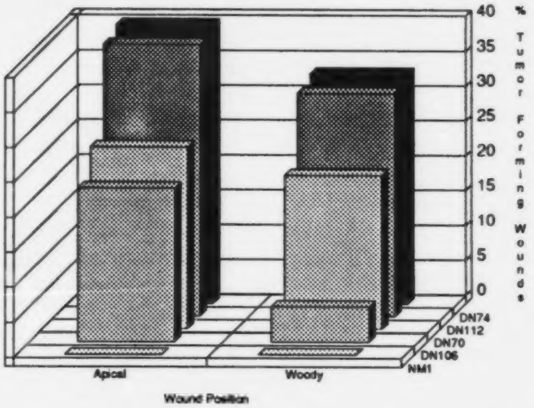


Figure 4. A208 (pTiT37 nopaline) tumor formation

Discussion

Plant-*Agrobacterium* interactions elicited markedly different responses at the clonal level. Factors influencing the initial survival of agrobacteria in wounds, as well as the subsequent binding and aggregation of bacteria at the wound site, can enhance or inhibit the tumor formation response. Other factors which can play a role are those such as the endogenous phytohormone levels in the stem at various points and the efficiency of DNA transfer from bacteria to the plant.

As well, there is evidence that virulence expression is activated specifically by the plant molecules acetosyringone and α-hydroxyacetosyringone (Stachel et al. 1985). The molecules occur at various levels in exudates of wounded and metabolically active plant cells, and the concentrations of these molecules may influence virulence induction and, hence, transformation efficiencies.

We have demonstrated that four hybrid clones of *Populus deltoides* x *nigra* are excellent hosts for *A. tumefaciens* strain A208, and that they form tumors in response to infection. The tumor genic tissue can subsequently be grown *in vitro* in the absence of added phytohormones. In contrast, hybrid *Populus nigra* x *maximowiczii* was demonstrated to be totally resistant to infection by this *Agrobacterium* strain. Differential interactions among *Agrobacterium* strains and cultivars has been shown in various other genera (Knauf et al. 1982, Miller et al. 1975, Owens and Cress 1985, Byrne et al. 1987). No evidence was produced for a systemic infection, as has been observed with *Crysanthemum* cultivars (Miller et al. 1975).

Spontaneous shoot regeneration from various tumor calli was infrequently observed *in vitro*. Crown gall tissues may regenerate spontaneously and this is mainly the case with nopaline-type crown gall tissues (Braun and Wood 1976). The detection of nopaline, while indicating possible transfer of the T-DNA to the plant cell and expression of the *nos* gene, is not actual evidence of T-DNA integration, because studies (Christou et al. 1986) indicate opine formation in normal callus and plant tissues as a result of arginine metabolism. DNA hybridization of T-DNA to transformed plant DNA is necessary to provide actual T-DNA integration.

Differential tumor formation response of host plants to nopaline and octopine strains has been reported (Otten 1982) and remains to be demonstrated for *Populus* spp. Poplar transformation technology, in combination with selective regeneration, has led to the recovery of *Populus* plants containing chimeric genes (Fillatti et al. 1987, Parsons et al. 1986). Evaluation of susceptibility of hybrid poplar clones of interest (such as natural simazine resistance) to various *Agrobacterium* strains will outline the engineered vectors that can be used for *in vitro* transformation systems with subsequent regeneration and propagation.

The present study provided a background for developing a gene transfer system in five *Populus* hybrids selected for their natural resistance to the herbicide simazine. The *in vivo* variations in susceptibility among clones and inoculation sites were analyzed. These poplar hybrid clones are amenable to manipulation *in vitro*, and provide a versatile system into which chimeric genes can be transferred and expressed using binary vectors.

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GENE AND RESTRICTION SITE MAPS OF DOUGLAS-FIR (*PSEUDOTSUGA MENZIESII* [MIRB.] FRANCO) AND RADIATA PINE (*PINUS RADIATA* D. DON) CHLOROPLAST GENOMES

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Abstract

We present chloroplast DNA gene and restriction site maps for Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) and radiata pine (*Pinus radiata* D. Don). DNA fragments were generated from restriction enzyme digests with *Pvu*II, *Sac*I/*Pvu*II, *Kpn*I, *Kpn*I/*Pvu*II, *Sma*I, and *Sma*I/*Pvu*II. Blots of these fragments were hybridized to PvuII fragments from Douglas-fir chloroplast DNA recovered from agarose gels, cloned chloroplast fragments from petunia and mung bean, and small gene fragments that code for known proteins in spinach, pea, and tobacco. Overlap filter hybridization was used to construct gene and restriction site maps. Chloroplast genomes of both species lack the large inverted repeat found in most land plants and algae, accounting for their relatively small size (approx. 120 kb). With the exception of a single 40 to 50 kb inversion, the linear arrangement of 32 genes in Douglas-fir and radiata pine appears to be identical.

Introduction

Chloroplasts contain DNA that codes for photosynthetic proteins and protein synthetic molecules such as RNA polymerases, transfer RNAs, ribosomal RNAs, and ribosomal proteins. Each chloroplast in a cell contains many, essentially homogeneous, copies of a covalently closed circular DNA molecule (cpDNA) that, in most species, is 120 to 160 kb in size (reviewed in Palmer 1985). Chloroplast DNA structure has been investigated in over 200 species of angiosperms from 33 families (reviewed in Whitfeld and Bottomley 1983, Gillham et al. 1985, Palmer 1985), a single gymnosperm (*Ginkgo biloba* L.) (Palmer and Stein 1986), ferns of the genus *Osmunda* (Stein et al. 1986), the liverwort *Marchantia polymorpha* (Ohyama et al. 1986), and algae (reviewed in Whitfeld and Bottomley 1983, Gillham et al. 1985, Palmer 1985). With few exceptions, cpDNAs contain a pair of large inverted repeats that encode the ribosomal RNA operon (rDNA), as well as other genes. In most angiosperms, the repeats are 20 to 30 kb in size and are separated by a large and a small single copy region. In addition to being fairly uniform in overall structure and size, cpDNAs tend to be highly conserved in gene arrangement among distantly related organisms.

Because of their relatively small size, chloroplast genomes are amenable to restriction-site mapping and gene mapping. Mapping is often done as a prerequisite to studies of gene structure and function, but is also useful in comparative studies of cpDNA structure and analysis of cpDNA restriction fragment length polymorphisms (RFLPs). Structural comparisons of cpDNAs are useful in constructing phylogenetic trees (Hosaka et al. 1984) and in determining the evolution of the chloroplast molecule itself (Palmer et al. 1987b). cpDNA RFLPs have been used in population genetics studies (Banks and Birky 1985, Holwerda et al. 1986, Wagner et al. 1987) and analyses of chloroplast inheritance (Neale et al. 1986).

Chloroplast genome structure of angiosperms has been well studied, but little is known about gymnosperms and, until recently, conifers were ignored. We present cpDNA gene and restriction site maps of two conifers, Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) and radiata pine (*Pinus radiata* D. Don). We show that these members of the Pinaceae lack the large inverted repeat common to most land plants and algae, and differ from one another by a major inversion.

Materials and Methods

Isolation of cpDNA

Chloroplast DNA was isolated from young Douglas-fir and radiata pine needles collected from two-year-old seedlings that were growing in the USDA Forest Service nursery in Placerville, CA. Needle samples were collected from approximately 30 seedlings of each species. Sucrose step-gradient centrifugation was used to isolate chloroplasts (Palmer 1986), except for modifications to the isolation buffer (0.35 M sorbitol, 50 mM Tris-HCl pH 8.0, 5 mM EDTA, 0.1% BSA w/v, 0.5% 2-mercaptoethanol, 10% polyethylene glycol-8000 w/v, and 0.1% PVP w/v) and wash buffer (0.35 M sorbitol, 50 mM Tris-HCl pH 8.0, 25 mM EDTA, 0.5% 2-mercaptoethanol).

Restriction enzyme digestion and Southern blotting

cpDNA fragments were generated from restriction enzyme digests with *Pvu*II, *Kpn*I, *Kpn*I/*Pvu*II, *Sac*I, *Sac*I/*Pvu*II, *Sma*I, and *Sma*I/*Pvu*II. Single and double digests were loaded into adjacent wells in 0.7% agarose gels, electrophoresed, stained with ethidium bromide, and photographed. Douglas-fir digests were run in lanes 1 to 7, and replicated in lanes 18 to 24, whereas radiata pine digests were run in lanes 9 to 15, and replicated in lanes 26 to 32 on each of two mapping gels. Douglas-fir *Pvu*II, *Sac*I, and *Kpn*I fragment sizes were estimated from gels run with λ-*Hind*III, λ-*Eco*RI, λ-*Sma*I, λ-*Sal*I, and øX174-*Hae*III fragments as molecular weight standards. On the mapping gels, all other fragment sizes were estimated using the Douglas-fir *Pvu*II, *Sac*I, and *Kpn*I fragments as molecular weight standards. DNA fragments were transferred to Zeta-bind filters (Cuno Inc., Meriden, CT) using a bidirectional Southern transfer (Maniatis et al. 1982). Once restriction-site maps had been completed, fragment sizes were adjusted slightly so that sizes of double-digest fragments summed to the corresponding single-digest size. Greater confidence was placed in estimates of fragment sizes that were in the linear portion of the log molecular weight/distance plot constructed for the mapping gels.

Douglas-fir *Pvu*II restriction fragment probes

Douglas-fir *Pvu*II cpDNA bands were cut from low melting temperature agarose gels and electroeluted in dialysis chambers. DNA was extracted with phenol:chloroform and ethyl ether and precipitated with ethanol. Because the 20.9 kb and 0.6 kb *Pvu*II bands were not successfully recovered from the gel, only 12 out of a total of 14 *Pvu*II bands were used in subsequent hybridizations. Because one of the bands turned out to be a doublet, these 12 bands represent 13 *Pvu*II fragments.

Petunia/mung bean cloned restriction fragment probes

Plasmids containing cloned restriction fragments from petunia (*Petunia hybrida*) and mung bean (*Vigna radiata* cv. Berken) were also used as probes to help map the regions covered by the missing 20.9 and 0.6 kb Douglas-fir *Pvu*II probes. The locations of the mung bean probes MB P18.8 and MB SP3.5 and petunia probe (PET P4.6) have been described graphically (Palmer and Stein 1986, Palmer et al. 1983). Recombinant plasmid DNA was isolated as described by Birnboim and Doly (1979).

Gene probes

Filters were also hybridized to 38 gene fragments (0.3 - 3.1 kb) that code for known proteins in spinach, pea, and tobacco (Tables 3 and 4). Thirty-five of the gene probes are cloned restriction fragments, whereas three of the probes (3'*rps7*, *rpo*A, and 3'*pet*D) are restriction fragments cut from 1% Sea-plaque low melting temperature agarose (FMC) gels. Details on recombinant plasmid construction, and the source of the gene probes, including species, restriction fragment, and gene location, are given by Jansen and Palmer (1987). Many of the clones contain sequences from more than one gene, or have sequences that are external to the gene(s) indicated in Tables 3 and 4.

Hybridizations and mapping

Recombinant plasmids were radioactively labelled with ^{32}P using a nick translation reagent kit (BRL, Gaithersburg, MD) according to the manufacturer's specifications. Hybridizations were performed according to Maniatis et al. (1982). Filters were washed at 65°C two times for about 30 min each in 2X SSC (0.3 M NaCl, 30 mM trisodium citrate) and 0.1% SDS, followed by two additional 30-min washes at 65°C in 2X SSC and 0.5% SDS prior to autoradiography. Restriction site maps for Douglas-fir and radiata pine were constructed by overlap hybridization (Palmer 1986).

Results and Discussion

Restriction fragment analysis

For each restriction enzyme combination, discrete bands could be seen when the gels were stained with ethidium bromide (Figure 1). Additional low molecular weight bands, not visible on the gels, could be located on autoradiograms because of their hybridization with one of the labelled probes (Tables 1-4). Contaminating nuclear DNA appeared as a diffuse smear in the *Kpn*I and *Sac*I digests, and as a high molecular weight smear in the *Pvu*II and *Sma*I digests. This reflects the relatively infrequent cleavage of nuclear DNA by *Pvu*II and *Sma*I. Some bands were stained with ethidium bromide much more strongly than others, suggesting that at least two fragments were comigrating. Overlap hybridization has confirmed that the following Douglas-fir bands contain DNA from two fragments each; *Pvu*II 11.3, *Sac*I 10.8 and 4.1, and *Sac*I/*Pvu*II 4.1. In radiata pine, two fragments are present in the following bands; *Sac*I/*Pvu*II 7.6 and 3.7, and *Kpn*I/*Pvu*II 13.8, and 5.8. Additionally, some pairs of radiata pine fragments with slightly different sizes appear as a single bright band on the gels. These include *Sma*I 31.8/30.7, and *Sma*I/*Pvu*II 2.9/2.8. Restriction-site mapping has identified other small fragments that are approximately equal in size, but comigration could not be detected visually. Summation of fragment sizes (including comigrating fragments) indicates a chloroplast genome size of about 121.4 kb for Douglas-fir and 119.6 for radiata pine (Figure 2).

Hybridizations with Douglas-fir *Pvu*II probes

When individual Douglas-fir *Pvu*II probes were hybridized to filter-bound Douglas-fir *Pvu*II fragments, a substantial number of the latter showed weak cross-hybridization (data not included). The number of cross-hybridizing fragments differed for each probe. *Pvu*II 2.1, for instance, hybridized weakly to eight other *Pvu*II fragments, whereas *Pvu*II 17.4 hybridized to only one. Cross-hybridization may occur for a number of reasons: 1) the fragments may represent different segments of a major repeat, such as the ubiquitous large inverted repeat; 2) dis-

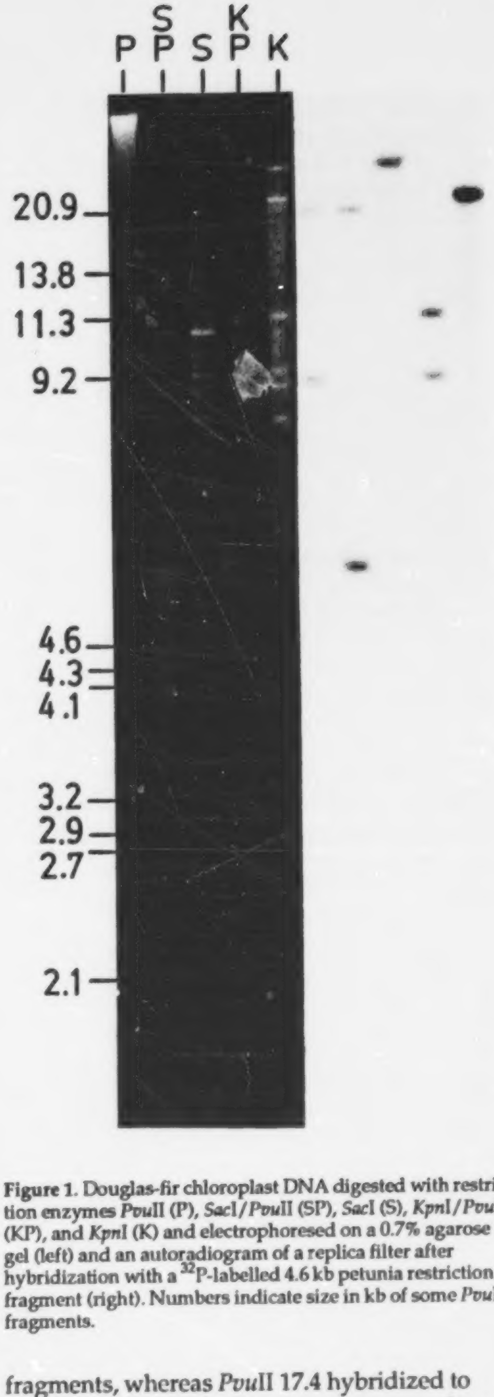

Figure 1. Douglas-fir chloroplast DNA digested with restriction enzymes *Pvu*II (P), *Sac*I/*Pvu*II (SP), *Sac*I (S), *Kpn*I/*Pvu*II (KP), and *Kpn*I (K) and electrophoresed on a 0.7% agarose gel (left) and an autoradiogram of a replica filter after hybridization with a ^{32}P-labelled 4.6 kb petunia restriction fragment (right). Numbers indicate size in kb of some *Pvu*II fragments.

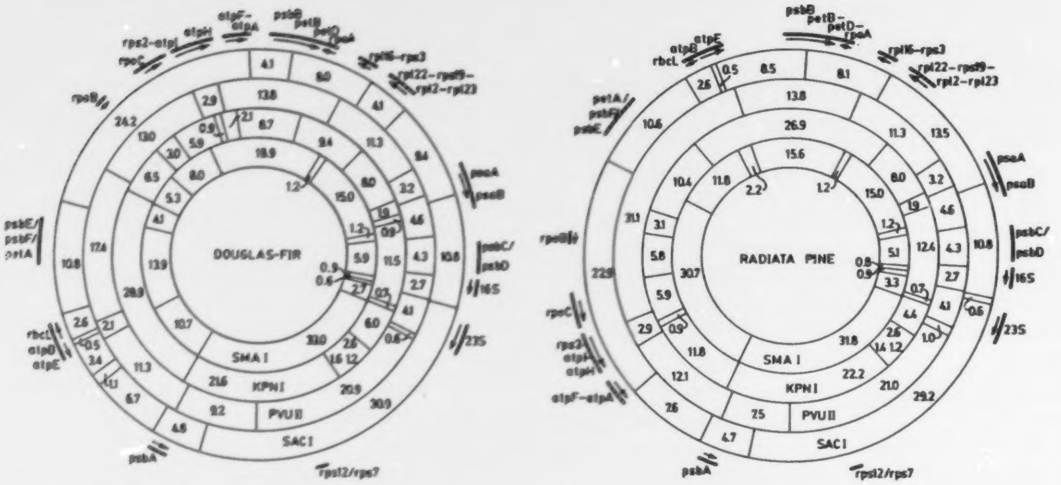

Figure 2. Restriction site and gene maps of Douglas-fir and radiata pine chloroplast genomes.

The linear order of genes separated by slashes could not be determined. The order of genes separated by dashes has been inferred from their arrangement in tobacco (Shinozaki et al. 1986). Transcriptional direction of *atp*H and *pet*D in Douglas-fir, and *psb*A in Douglas-fir and *Pinus radiata*, has been assigned based on differential hybridization of 5′ and 3′ gene probes (Tables 3 and 4). Transcriptional directions of other genes and the location and transcriptional directions of 16S and 23S rRNA have been assigned by analogy to other organisms (described in text). Dark bands represent the lengths of gene probes used in hybridizations, rather than the lengths of the genes themselves, except for 16S and 23S rRNA, for which no gene probes were used.

persed repetitive sequences may occur on different cpDNA fragments (Bowman and Dyer 1986, Palmer et al. 1987a, 1987b); 3) the probe may be contaminated with DNA from the fragment to which it hybridizes; or 4) the probe may contain sequences homologous to repetitive nuclear (Scott and Timmis 1984) or mitochondrial (Stern and Palmer 1984) DNA fragments that comigrate with the cpDNA fragment which appears to participate in the hybridization.

The notion that different Douglas-fir *Pvu*II fragments have extensive homologous coding regions can be ruled out, based on the weakness of the cross-hybridizations and lack of corroborating gene probe hybridization data (Tables 3-4). The patterns of cross-hybridization were particularly noticeable between fragments of similar size, suggesting that DNA contamination occurred when adjacent bands were cut from the gel. The similar hybridization patterns of *Pvu*II 2.7 and 2.9 (Tables 1-2) are probably due to cross-contamination between the probes. Cross-hybridizations between fragments that were not adjacent to one another on the gel also occurred. In most cases, small probes hybridized to larger fragments. When the larger fragments were used as probes, however, the expected reciprocal hybridizations generally did not occur. Six of the Douglas-fir *Pvu*II probes, for example, hybridized to *Pvu*II 17.4, but when *Pvu*II 17.4 was used as a probe, it hybridized reciprocally to only one (data not included). This pattern of cross-hybridization is likely to have resulted from contamination of the smaller probes with degraded DNA from the larger fragments.

The strongest cross-hybridization between Douglas-fir *Pvu*II fragments involved *Pvu*II 3.2 and *Pvu*II 2.7 when *Pvu*II 3.2 was used as the probe (Table 1). On the other hand, when *Pvu*II 2.7 was used as the probe, it hybridized weakly to *Pvu*II 3.2 (not included in Table 1). *Pvu*II 3.2 also hybridized to nearly all of the fragments that hybridized to the *Pvu*II 2.7 probe (Tables 1-2). In every case, however, the *Pvu*II 3.2 probe hybridized to other fragments that did not hybridize to the *Pvu*II 2.7 probe. For this reason, we believe that the *Pvu*II 3.2 probe was contaminated with DNA from *Pvu*II 2.7, although the source of the contamination is unknown.

Additional cross-hybridizations may result from homologies between dispersed repetitive elements on separate Douglas-fir *Pvu*II restriction fragments. Cross-hybridization between cloned Douglas-fir *Xba*I cpDNA probes and filter-bound *Xba*I cpDNA fragments (unpublished data) suggests that some of the cross-hybridizations are caused by short-dispersed repetitive sequences. The *Xba*I clones are not subject to the same DNA contamination problems that plague probes cut from gels.

We believe that many of the weak hybridizations with the *Pvu*II probes were caused by DNA contamination or the presence of dispersed repetitive sequences. Because this information does not contribute to the overlap hybridization mapping strategy, many weak hybridizations were not included in Tables 1 and 2. Somewhat arbitrary criteria were necessarily used in deciding that weak hybridization was not caused by limited fragment overlap or sequence divergence between Douglas-fir and radiata pine. These decisions, however, were supported by information from the cloned heterologous probes, which are not subject to problems of DNA cross-contamination, and presumably do not contain sequences homologous to dispersed repetitive sequences in Douglas-fir or radiata pine.

Nearly all of the filter-bound fragments hybridized strongly to at least one of the Douglas-fir *Pvu*II probes (Tables 1-2). We assumed that non-hybridizing fragments are located in regions covered by the missing 20.9 kb and 0.6 kb *Pvu*II probes. This assumption is supported by subsequent hybridizations with petunia/mung bean restriction fragment probes (Tables 1-2) and gene probes (Tables 3-4), except for: Douglas-fir *Kpn*I fragments 2.6, 1.6, and 1.2; Douglas-fir *Kpn*I/*Pvu*II fragments 2.6 and 1.6; radiata pine *Kpn*I fragments 2.6, 1.4, and 1.2; radiata pine *Kpn*I/*Pvu*II fragment 2.6; and, a Douglas-fir 5.1 kb *Sac*I fragment, for which there was no corroborating evidence. The single- and double-digest *Kpn*I fragments did not hybridize to any probe, and were mapped on to the region covered by the Douglas-fir 20.9 kb probe by process of elimination. We concluded that the Douglas-fir 5.1 kb *Sac*I band represents a highly repetitive nuclear or mitochondrial sequence because it is absent from gels that were run with more highly purified cpDNA (not illustrated).

Some of the Douglas-fir probes also hybridized to places on the filters where no band could be seen on the gel. This was true for both Douglas-fir and radiata pine. Douglas-fir probes *Pvu*II 2.7 and *Pvu*II 4.1, for example, hybridized strongly to a spot on the filters corresponding to a fragment size of about 6.9 kb. This could result from hybridizations of the probe with incomplete cpDNA digestion products or with highly repeated homologous nuclear or mitochondrial sequences. Because *Pvu*II 2.7 and *Pvu*II 4.1 are adjacent to one another in both Douglas-fir and radiata pine, incomplete digestion could result in a fragment of about 6.9 kb.

Petunia/mung bean restriction fragment hybridizations

Initially, portions of the Douglas-fir and radiata pine genomes could not be mapped because we did not have a complete set of Douglas-fir *Pvu*II fragments to use as probes (Tables 1-2). Three heterologous fragments (MB P18.8, MB SP3.5, and PET 4.6) were used for mapping these gaps. Each of these fragments hybridized to both of the missing Douglas-fir fragments (*Pvu*II 20.9 and 0.6), as well as other fragments whose location had already been determined (Tables 1-2).

The location of the rDNA operon in Douglas-fir and radiata pine was determined by hybridization of MB P18.8 and MB SP3.5, which cover regions that include, and flank, the rDNA operon in petunia and mung bean (Palmer and Stein 1986, Palmer et al. 1983). The location and orientation of the rDNA operon was further elucidated by the presence in Douglas-fir and radiata pine of two highly conserved *Pvu*II fragments (approx. 4.1 kb and 2.7 kb). These fragments contain the 16S and 23S rRNA genes in the same orientation in almost all vascular plants (Palmer and Stein 1982, Palmer and Stein 1986).

Gene probe hybridizations

Because of their small size, the gene probes tended to hybridize to single fragments in each of the digests (Tables 3-4). A notable exception was 3′*rbc*L, which hybridized to two distinct

Table 1. Hybridization of Douglas-fir (DF) and petunia (PET) cpDNA probes with filter-bound Douglas-fir cpDNA restriction fragments.

		Douglas-fir filter-bound cpDNA hybridized[2]						
Species	**Probe cpDNA[1]**	***Pvu*II**	***Sac*I**	***Sac*I/*Pvu*II**	***Kpn*I**	***Kpn*I/*Pvu*II**	***Sma*I**	***Sma*I/*Pvu*II**
		Fragment Size (kb)						
DF	20.9	?	?	?	?	?	?	?
DF	17.4	17.4	24.2,10.8,2.6	10.8,4.7,4.3,1.9	28.9,6.5	16.4,1.0	13.9,5.3,4.1	12.2,4.1,1.1
DF	13.8	13.8	24.2,8.0,4.1	6.1,4.1,3.6	9.4,8.7,2.1	8.7,3.6,1.5	18.9	13.8
DF	13.0	13.0	24.2	13.0	6.5,5.9,3.0	5.5,4.5,3.0	18.9,8.0,5.3	8.0,4.2,0.8
DF	11.3	11.3	9.4,8.0,6.7,4.8, 4.1,3.4,1.1	6.7,5.3,4.1,2.5, 1.9,1.1	28.9,21.6,9.4,8.0	10.4,5.8,5.5,0.9	33.0,18.9,15.0, 10.7,1.2	10.3,8.7,1.4, 1.2,1.0
DF	9.2	9.2	30.9,4.8	5.4,3.8	21.6	9.2,5.8	33.0	9.2
DF	4.6	4.6	10.8,9.4	3.7,0.9	11.5,1.9,0.9	2.5,1.2,0.9	15.0,5.9,1.2	3.1,1.2
DF	4.3	4.3	10.8	4.3	11.5	4.3	5.9,2.7	4.3,2.7
DF	4.1	4.1	30.9,10.8	4.0	11.5,6.0,0.7	4.3,2.0,1.4,0.7	33.0,2.7	2.7,1.3
DF	3.2	3.2,2.7^{a}	10.8^{a},9.4	3.2,2.7^{a}	11.5^{a},8.0,5.9^{b}, 1.9	0.7	15.0,5.9^{a}, 0.9^{a}	3.2,2.9^{b}, 1.3^{a},0.9^{a}
DF	2.9	2.9	24.2	2.9	5.9,2.1,0.9	2.7^{a},1.4,0.9,0.6	18.9	2.9
DF	2.7	2.9_{b},2.7	10.8	2.9^{b},2.7	11.5,5.9^{b}	2.7,1.4^{b},0.9^{b},0.6^{b}	5.9,0.9	2.9^{b},1.3,0.9
DF	2.1	2.1	3.4,2.6,0.5	0.9,0.7,0.5	28.9	2.1	13.9,10.7	1.7
DF	0.6	?	?	?	?	?	?	?
MB	P18.8	20.9,4.1,2.7, 0.6	30.9,10.8	20.9,4.3^{c},4.0, 2.7,0.6	21.6,11.5,6.0,0.7	11.5,4.3^{c},4.0^{c}, 2.7^{c},2.0,1.4,0.7,0.6	33.0,5.9,2.7, 0.9	20.9,4.3^{c},2.7, 1.3,0.9,0.6
MB	SP3.5	20.9,4.1,0.6	30.9	20.9,4.0,0.6	6.0	4.0,1.4,0.6	33.0	20.9,1.3,0.6
PET	P4.6	20.9,9.2	30.9	20.9,5.4	21.6	11.5,9.2	33.0	20.9,9.2

[1] Probe cpDNA is designated as P for *Pst*I, S for *Sal*I, or SP for *Sal*I/*Pst*I, followed by fragment size in kb. All Douglas-fir probes are *Pvu*II fragments. MB SP3.5 is homologous to one end of MB P18.8.

[2] Non-hybridizing cpDNA fragments which could be seen on the gel include *Pvu*II 20.9 and 0.6; *Sac*I/*Pvu*II 20.9 and 1.0; *Kpn*I 2.6, 1.6, and 1.2; *Kpn*I/*Pvu*I 11.5, 4.0, 2.6, and 1.6; and *Sma*I/*Pvu*II 20.9.

? Fragment was not used as a probe.

[a] Hybridization probably results from contamination of the probe with DNA from *Pvu*II 2.7.

[b] Hybridization probably results from contamination of the probe with DNA from *Pvu*II 2.9.

[c] Weak hybridization.

Table 2. Hybridizations of Douglas-fir (DF), mung bean (MB), and petunia (PET) cpDNA probes with filter-bound radiata pine cpDNA restriction fragments.

		Radiata pine filter-bound cpDNA hybridized[2]						
Species	**Probe cpDNA[1]**	***Pvu*II**	***Sac*I**	***Sac*I/*Pvu*II**	***Kpn*I**	***Kpn*I/*Pvu*II**	***Sma*I**	***Sma*I/*Pvu*II**
		Fragment Size (kb)						
DF	20.9	?	?	?	?	?	?	?
DF	17.4	31.1	22.9,10.6(10.8),2.6	16.5,10.6,4.3,2.6	26.9,10.4	10.4,7.3,4.3	30.7(31.8)[a],11.8,5.1	16.7,11.8,4.3
DF	13.8	13.8,12.1	22.9,8.5,8.1	7.6,6.2,3.5	26.9,11.8	13.8,11.2	30.7(31.8),15.6(15.0)	13.8,11.1
DF	13.0	31.1	22.9	16.5	5.9,5.8,3.1	5.8,4.5,3.1	30.7(31.8)	16.7
DF	11.3	13.8,12.1, 11.3	13.5,8.5,8.1,7.6, 4.7	9.4,7.6,1.9,1.0	26.911.8,8.0	13.8,11.2,5.8, 5.5,0.9	31.8 and/or 30.7, 15.6 and/or 15.0,1.2	13.8,11.1,8.7, 1.4,1.2,1.0
DF	9.2	7.5,21.0, 11.3,13.8	29.2,13.5,4.7	3.8,3.7	26.9,22.2	13.8,7.5,5.8	31.8(30.7), 15.0 and/or 15.6	7.5
DF	4.6	4.6	13.5,10.8(10.6)	3.7,0.9	12.4,1.9	3.4,1.2	15.0(15.6),1.2	3.1,1.2
DF	4.3	4.3	10.8(10.6)	4.3	12.4	4.3	5.1	4.3
DF	4.1	4.1	29.2,0.6	3.4,0.6	12.4,4.4,0.7	2.0,1.4,0.7	31.8(30.7),3.3	2.8,1.3
DF	3.2	3.2,2.7[b]	13.5,10.8[b](10.6)	3.2,2.7[b]	12.4[b],8.0, 1.9	2.7[b],2.5,0.7	15.0(15.6),0.9[b], 0.8[b]	3.2,0.9[b],0.8[b]
DF	2.9	2.9	22.9	2.9	12.4[b],5.9, 0.9	1.4,0.9,0.6	30.7(31.8),15.0 and/or 15.6,0.9[b],0.8[b]	2.9,0.9[b],0.8[b]
DF	2.7	2.7	10.8(10.6)	2.7	12.4	2.7	5.1,0.9,0.8	0.9,0.8
DF	2.1	31.1,13.8	8.5,0.5	0.9,0.5	26.9	7.3	2.2	2.2
DF	0.6	?	?	?	?	?	?	?
MB	P18.8	21.0,4.1,2.7	29.2,10.8,0.6	21.0, 3.4,2.7,0.6	12.4,4.4,0.7	2.7,2.0,0.7	31.8(30.7),5.1,3.3,0.8	2.8(2.9),0.8
MB	SP3.5	4.1,1.0	29.2	21.0,3.4,1.0	4.4	1.4,1.0	31.8(30.7)	21.0,1.3,1.0
PET	P4.6	21.0	29.2	21.0	22.2	13.8	31.8(30.7)	21.0

[1] Probe DNA is designated as P for *Pst*I, S for *Sal*I, or SP for *Sal*I/*Pst*I, followed by fragment size in kb. All Douglas-fir probes are *Pvu*II fragments. MB SP3.5 is homolgous to one end of MB P18.8.

[2] Non-hybridizing cpDNA fagments which could be seen on the gel include *Pvu*II 1.0; *Sac*I/*Pvu*II 21.0; *Kpn*I 2.6, 1.4, and 1.2; *Kpn*I/*Pvu*II 2.6, and 1.0; and *Sma*I/*Pvu*II 21.0.

? = Fragment was not used as a probe.

[a] Fragments in parentheses cannot be distinguished on the autoradiogram from the fragment preceding it in the table. Hybridization of probe to the fragment not enclosed in parentheses is consistent with Figure 2.

[b] Hybridization probably results from contamination of the probe with DNA from *Pvu*II 2.7.

Table 3. Hybridizations of cpDNA gene probes from spinach, pea, and tobacco with filter-bound Douglas-fir cpDNA restriction fragments.

	Douglas-fir filter-bound cpDNA hybridized							
Gene Probe	*Pvu*II	*Sac*I	*Sac*I-*Pvu*II	*Kpn*I	*Kpn*I-*Pvu*II	*Sma*I	*Sma*I-*Pvu*II	References
				Fragment Size (kb)				
3'rps12-5'rps7	29.9	30.9	20.9	21.6	11.5	33.0	20.9	a
3'rps7								a
rp123-5'rp12		4.1	4.1	9.4	5.8		8.7	b
3'rp12-rps19-5'rp122	11.3	4.1	4.1	9.4	5.8	15.0	8.7	b
3'rp122-5'rps3								b
3'rps3-5'rp116	11.3	8.0	1.9	9.4	5.8	18.9		b
3'rp116-rp114-5'rps8								b
3'rps8-infA								b,c
rps11								c
rpoA		8.0	6.1	9.4	3.6	18.9	13.8	c
3'petD	13.8	8.0	6.1	9.4,8.7	8.7,3.6	18.9	13.8	d
5'petD	13.8	8.0	6.1	8.7	8.7	18.9	13.8	d
petB	13.8	8.0	6.1	8.7	8.7	18.9	13.8	d
3'psbB				8.7	8.7	18.9	13.8	d
5'psbB	13.8	4.1	4.1	8.7	8.7	18.9	13.8	d
5'psbE	17.4	10.8	10.8	28.9	16.4	13.9	12.2	e
3'psbE-psbF	17.4	10.8	10.8	28.9	16.4	13.9	12.2	e
3'petA	17.4	10.8	10.8	28.9	16.4	13.9	12.2	f
5'petA	17.4	10.8	10.8	28.9	16.4	13.9	12.2	f
3'rbcL	17.4,11.3	6.7		28.9	16.4,10.4	13.9,10.7	12.2,10.3	g
rbcL	17.4	2.6	1.9	28.9	16.4	13.9	12.2	g
5'rbcL		2.6		28.9	16.4	13.9	12.2	g
atbB	2.1	3.4	0.9	28.9	2.1	13.9	1.7	g
atpE				28.9		10.7		h
psaA	3.2,4.6	9.4	3.2,0.9	8.0,1.9	1.2,0.7	15.0	3.2,3.1	i
psaB	4.6	10.8	3.7	11.5,0.9	2.5,0.9	15.0	3.1	i
3'psbC	4.3	10.8	4.3	11.5	4.3	5.9	4.3	i
3'psbD-5'psbC	4.3	10.8	4.3	11.5	4.3	5.9	4.3	j
5'psbD	4.3	10.8	4.3	11.5	4.3	5.9	4.3	j
rpoB	13.0		13.0	6.5	5.5	8.0	8.0	k
rpoC	13.0	24.2	13.0	5.9	4.5	8.0	8.0	l
rps2-atpI-5'atpH	2.9	24.2	2.9	5.9	1.4	18.9	2.9	m
3'atpH	2.9	24.2	2.9	2.1		18.9	2.9	n
atpF-5'atpA	13.8	24.2	3.6	8.7	8.7	18.9	13.8	o
3'atpA	13.8	24.2	3.6	8.7	8.7	18.9	13.8	o
rps16								p
5'psbA	11.3	4.8	1.0	21.6	0.9	33.0	1.0	q
3'psbA	9.2	4.8	3.8	21.6	9.2	33.0	9.2	q

[a] Fromm et al. (1986).

[b] Shinozaki et al. (1986b)

[c] Sijben-Muller et al. (1986).

[d] Heinemeyer et al. (1984).

[e] Herrmann et al. (1984).

[f] Willey et al. (1984).

[g] Zurawski et al. (1986a, 1986b).

[h] Zurawski et al. (1982).

[i] Alt et al. (1984).

[j] Rasmussen et al. (1984).

[k] Ohme et al. (1986).

[l] Cozens and Walker (1986).

[m] Cozens et al. (1986).

[n] Huttly and Gray (1984, unpublished data).

[o] Westhoff et al. (1985).

[p] Shinozaki et al. (1986a).

[q] Oishi et al. (1984).

Table 4. Hybridizations of cpNDA gene probes from spinach, pea, and tobacco with filter-bound radiata pine cpDNA restriction fragments

	Radiata pine filter-bound cpDNA hybridized							
Gene Probe	*Pvu*II	*Sac*I	*Sac*I-*Pvu*II	*Kpn*I	*Kpn*I-*Pvu*II	*Sma*I	*Sma*I-*Pvu*II	References
	Fragment Size (kb)							
3'rps12-5'rps7	21.0	29.2	21.0	22.2	13.8	31.8(30.7)[*]	21.0	a
3'rps7								a
rpl23-5'rp12		13.5	9.4	26.9	5.8		8.7	b
3'rp12-rps19-5'rp122	11.3	13.5	9.4	26.9	5.8		8.7	b
3'rp122-5'rps3								b
3'rps3-5'rp116	11.3	8.1	1.9	26.9	5.8	15.6		b
3'rp116-rp114-5'rps8								b
3'rps8-infA								b,c
rps11								c
rpoA		8.1		26.9	13.8	15.6	13.8	c
3'petD	13.8	8.1	6.2	26.9	13.8	15.6	13.8	d
5'petD	13.8	8.1	6.2	26.9	13.8	15.6	13.8	d
petB	13.8	8.1	6.2	26.9	13.8	15.6	13.8	d
3'psbB								d
5'psbB	13.8	8.5	7.6	26.9	13.8	15.6	13.8	d
5'psbE	31.1	10.6	10.6	10.4	10.4	11.8	11.8	e
3'psbE-psbF	31.1	10.6	10.6	10.4	10.4	11.8	11.8	e
3'petA	31.1	10.6	10.6	10.4	10.4	11.8	11.8	f
5'petA	31.1	10.6	10.6	10.4	10.4	11.8	11.8	f
3'rbcL	12.1			26.9		31.8 and/or 30.7		g
rbcL	31.1	2.6	2.6	26.9	7.3	11.8	11.8	g
5'rbcL				26.9				g
atbB	31.1	8.5,2.6,0.5	2.6,0.9,0.5	26.9	7.3	2.2	2.2	g
atpE				26.9		15.6		h
psaA	4.6,3.2	13.5	3.2	8.0,1.9	2.5,1.2,0.7	15.0	3.2,3.1	i
psaB	4.6	10.8	3.7	12.4	3.4	15.0	3.1	i
3'psbC	4.3	10.8	4.3	12.4	4.3	5.1	4.3	i
3'psbD-5'psbC	4.3	10.8	4.3	12.4	4.3	5.1	4.3	j
5'psbD	4.3	10.8	4.3	12.4	4.3	5.1	4.3	j
rpoB		22.9	16.5	5.8	5.8	30.7(31.8)	16.7	k
rpoC		22.9	16.5	5.9	4.5	30.7(31.8)	16.7	l
rps2-atpI-5'atpH	2.9	22.9	2.9	5.9	14.	30.7(31.8)	2.9(2.8)	m
3'atpH							2.9(2.8)	n
atpF-5'atpA	12.1	22.9	3.5	11.8	11.2		11.1	o
3'atpA	12.1	22.9	3.5	11.8	11.2		11.1	o
rps16								p
5'psbA	12.1	4.7	1.0	22.2	0.9	31.8(30.7)	1.0	q
3'psbA	7.5	4.7	3.7	22.2	7.5	31.8(30.7)	7.5	q

[*] Fragments in parentheses cannot be distinguished on the autoradiogram from the fragment preceding it in the table. Hybridization of the probe to the fragment not enclosed in parentheses is consistent with Figure 2.

[a] Fromm et al. (1986).
[b] Shinozaki et al. (1986b).
[c] Sijben-Muller et al. (1986).
[d] Heinemeyer et al. (1984).
[e] Herrmann et al. (1984).
[f] Willey et al. (1984).
[g] Zurawski et al. (1986a, 1986b).
[h] Zurawski et al. (1982).
[i] Alt et al. (1984).
[j] Rasmussen et al. (1984).
[k] Ohme et al. (1986).
[l] Cozens and Walker (1986).
[m] Cozens et al. (1986).
[n] Huttly and Gray (1984, unpublished data).
[o] Westhoff et al. (1985).
[p] Shinozaki et al. (1986a).
[q] Oishi et al. (1984).

locations in Douglas-fir (Table 3). This probe hybridized weakly to fragments that hybridized with *rbc*L and 5′*rbc*L, and strongly to fragments that lie between *atp*E and *psb*A (Table 3, Figure 2). The *rbc*L probe contains the 0.7 kb segment downstream of the *rbc*L termination codon in pea, in addition to the 3′ end of *rbc*L. We believe the *rbc*L portion of the probe hybridized to the location where *rbc*L is found in Douglas-fir, and the remainder of the probe hybridized to the area between *atp*E and *psb*A, where the *rbc*L probe restriction fragment is located in pea (Palmer 1985). In radiata pine, 3′*rbc*L did not hybridize detectably to the same location as *rbc*L and 5′*rbc*L. We believe the unusual hybridization pattern of 3′*rbc*L in radiata pine results from detectable hybridization of only the non-*rbc*L part of the probe. Rearrangements between pea and radiata pine presumably account for hybridization of the probe to two separate regions of the radiata pine genome.

The degree of hybridization varied greatly; in fact, it was impossible to detect hybridization with some of the probes (Tables 3-4). Differential hybridization of separate 5′ and 3′ gene fragments allowed us to determine transcriptional direction for the Douglas-fir and radiata pine *psb*A gene, and the Douglas-fir *pet*D and *atp*H genes (Figure 2). In some cases, the linear order of genes within tightly-linked gene clusters could be determined by differential hybridization of gene probes. Because the orientation of these genes is highly conserved, we were able to infer gene orientation in Douglas-fir and radiata pine by analogy to tobacco (Shinozaki et al. 1986b). In a few cases, both the linear order of genes and transcriptional direction were inferred based on adjacent hybridizations of gene probes (Figure 2).

Douglas-fir and radiata pine lack the large inverted repeat

Restriction site and gene maps of Douglas-fir and radiata pine indicate that both chloroplast genomes lack the large inverted repeat typical and most land plants and algae (Figure 2). The vast majority of gene probes hybridized strongly and specifically to a single restriction fragment in each digest, indicating that gene duplication is unlikely. There was only a single case where a gene probe (3′*rbc*L) hybridized to fragments that are located on separate parts of the molecule (Tables 3-4), indicating that if duplications exist, gene copies would have to be fairly close together. The hybridization patterns of MB P18.8 and MB SP3.5 are consistent with a single copy of the rDNA operon, and the highly conserved rDNA containing *Pvu*II fragments (4.1 and 2.7 kb) visually appear to be present in unimolar amounts.

A major inversion between Douglas-fir and radiata pine

Gene mapping shows that Douglas-fir and radiata pine differ by a single major inversion. *Atp*A and *atp*E are located near the ends of the inverted segment, whereas *psb*B and *psb*A flank the inversion on the uninverted portion of the molecule (Figure 2). In Douglas-fir the *Sac*I 4.1 kb and *Pvu*II 11.3 fragments appear to contain the endpoints, indicating that the size of the inversion is between 39.0 and 54.4 kb. Based on gene probe and *Pvu*II fragment hybridizations, the size of the inversion in radiata pine is between 37.5 and 53.7 kb.

Conclusions

Douglas-fir and radiata pine cpDNAs lack the large inverted repeat common to most land plants and algae. This accounts for their relatively small chloroplast genome sizes (approx. 120 kb). Among the angiosperms, loss of the inverted repeat has been found in only a few legumes, although genome structure has been studied in over 200 species in 33 families. The inverted repeat has been found in the ancient gymnosperm *Ginkgo biloba* (Palmer and Stein 1986), indicating that loss of the repeat probably occurred after the divergence of the Pinaceae from the Ginkgoales. It will be interesting to see which other gymnosperms lack the inverted repeat. This information will shed light on the phylogenetic relationships among gymnosperms, and also on the role of the inverted repeat in chloroplast DNA evolution.

The linear arrangement of 32 genes in Douglas-fir and radiata pine appears to be identical, except for a single inversion of roughly 40 to 50 kb. One end of the inversion lies downstream from *atp*A. Intriguingly, this location is also the endpoint of major inversions in pea (Palmer et al. 1985), mung bean (Palmer and Thompson 1982, reviewed in Palmer 1985), *Oenothera* (Herrmann et al. 1983), wheat (Howe et al. 1983; Quigley and Weil 1985), and lettuce (Jansen and

Palmer 1987). In lettuce (Jansen and Palmer 1987) and wheat (Howe 1985, Quigley and Weil 1985), the inversion endpoint has been localized more specifically to a space between tRNA genes. The endpoint of the inversion between Douglas-fir and radiata pine may be in a similar location.

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LACK OF ONE rDNA REPEAT AND DUPLICATION OF *psb*A IN THE CHLOROPLAST GENOME OF LODGEPOLE PINE (*PINUS CONTORTA* DOUGL.)

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Abstract

Chloroplast DNA from lodgepole pine *Pinus contorta* Dougl. was studied by Southern blot analysis. A heterologous *psb*A probe was found to hybridize equally strongly to two cpDNA fragments in each enzyme digest. The two *psb*A hybridizing *Bam*HI fragments were cloned and, by partial sequencing, the presence of one *psb*A gene on each fragment was confirmed. The prime characteristic of chloroplast genomes is the inverted repeat structure, a duplicated segment which contains genes for rRNA, tRNA, and ribosomal proteins. However, 16S and 23S rDNA probes hybridized to only one lodgepole pine cpDNA fragment each per enzyme digest. The unique 16S rDNA-containing *Bam*HI fragment was cloned and restriction mapped. The 16S rRNA gene was located within the *Bam*HI fragment, which also contained the *psb*C and *psb*D genes. We conclude that the structure of the chloroplast genome of *P. contorta* deviates from that of other higher plants in at least two ways; the *psb*A gene is duplicated and one of the rRNA-encoding repeats is absent.

Introduction

The chloroplast (cp) genome of plants encodes and expresses a significant part of the genetic information needed for photosynthesis. Data describing the structure of the cp genome from a large number of angiosperm plants, some mosses, and several algae (Palmer 1985) are now available. Analysis of complete nucleotide sequence data from *Nicotiana tabacum* (Shinozaki et al. 1986) and *Marchantia polymorpha* (Ohyama et al. 1986) chloroplast genomes indicate high levels of genomic homogeneity exist for both species. Specifically, little variation was observed in gene content, the presence of inverted repeats, and single copy regions. This contrasts with the variability observed in gene orders and genomic sizes (Palmer 1985, Rachaix 1985). However, little is known about the cp genome of coniferous plants.

Conifers represent a major and phylogenetically distinct group of plants. Advanced evolutionary age--more than 100 million years for pines (Mirov 1967)--and the pronounced conservatism of conifers, make them interesting objects for various phylogenetic and biochemical studies. *Pinus contorta* Dougl. is widespread in North America, with a range extending from the Yukon (lat. 64°) to California (lat. 31°) (Critchfield and Little 1966).

We studied the structure of chloroplast genomes from *P. contorta* individuals in order to define differences which may have phylogenetic and physiological implications. The most characteristic and conserved structural feature of chloroplast genomes are the inverted repeats (IR). These two identical segments, which separate the circular molecule into a large and a small single copy region (LSC and SSC), contain genes for rRNA, tRNA, and ribosomal proteins. The D1-protein, encoded by the *psb*A gene, is believed to constitute the reaction center of photosystem II, together with the D2-protein. The D1-protein also displays interesting properties concerning turnover and susceptibility to photodestruction (Kyle 1985). Restriction maps over the cp genomes from a large number of higher plants have invariably shown the *psb*A gene to be present as a single copy gene in the LSC region of the chloroplast genome (Ohyama et al. 1986, Palmer 1985, Rochaix 1985, Shinozaki et al. 1986). In contrast, the *psb*A gene is duplicated in the green alga *Chlamydomonas rein-*

hardtii (Erickson et al. 1984) and in the chromophytic alga *Olistodiscus luteus* (Reith and Cattolico 1986), the two copies being located at identical positions within the inverted repeats. In this paper we report that the *psb*A gene is duplicated on the cp genome of lodgepole pine and that this genome also lacks one of the rRNA-encoding inverted repeats.

Materials and methods

Plant material, bacterial strains, vectors, and probes

Half-sib families of lodgepole pine (Tom Creek Tower, Canada, lat. 60°17′, long. 129°00′, alt. 950 m) were grown in a climate chamber to four months of age before harvest. Plasmids used for the library and for subclonings were pUC9 and pUC19 (Yanisch-Perron et al. 1985). Subclonings for sequencing were made in M13mp18 (Sanger et al. 1977). The *Escherichia coli* strains used were DH5α, a derivative of DH1 (Hanahan 1983), for plasmids and PC2495, a derivative of JM101 (Yanisch-Perron et al. 1985), for M13 phages. The *psb*A specific probe used in this study was an internal 769 bp *Pst*I/*Xba*I fragment from the spinach clone pPSII32/1 (equivalent to amino acids 87 to 341), and the *psb*C and *psb*D probes were the spinach clones pPSII44 and pPSII32/2 (Bohnert and Löffelhardt 1982), respectively, all kindly provided by Prof. R.G. Herrmann. The 16S and 23S rDNA specific probes were derived from the 6.6 kb *Pst*I insert of the clone pAN4 (Tomioka et al. 1981) which was kindly provided by Prof. M. Sugiura.

DNA techniques

The method to extract cpDNA from pines has been described elsewhere (Szmidt et al. 1987). DNA was digested with restriction enzymes according to standard procedures. Agarose gel electrophoresis and blot transfers to Hybond-N membrane (Amersham Inc.) were made according to Southern (1975). The DNA size marker used was the BRL 1 kb ladder which comprises a ladder of 1018 bp increments, ranging from 1018 to 12 216 bp. Radioactive probes were prepared using random priming procedures (Feinberg and Vogelstein 1983). Heterologous filter hybridizations were performed at 58°C according to Maniatis et al. (1982). To remove the probe prior to rehybridization, filters were washed in 0.1X SSC, 0.1% SDS at 95°C for 10 min.

A cpDNA library was made by ligating 1 μg *Bam*HI-digested cpDNA to pUC9 and transforming DH5α cells. The transformation yielded 2500 ampicillin resistant lac$^-$ colonies. To identify recombinant clones containing the *psb*A genes, colony hybridization was done using the spinach *psb*A probe. The isolated *psb*A-positive clones pPC121 and pPC932 and the 16S rDNA-containing clone pPC28 were restriction mapped by conventional methods. Sequencing was performed by the standard M13 procedure (Sanger et al. 1977). The DNA sequences obtained were processed by the GENEUS computer program (Harr et al. 1986).

Results

Southern hybridization of *psb*A to chloroplast DNA

In Southern blot analysis of cpDNA from *P. contorta*, a heterologous *psb*A-specific probe hybridized equally strongly to two fragments in each enzyme digest (Figure 1b). These fragments were *Eco*RI 4.9 and 3.7 kb, *Sac*I 4.8 and 4.3 kb, and *Bam*HI 3.8 and 2.9 kb (Figure 1a-b). This observation strongly suggests a gene duplication of *psb*A.

Isolation of the two *psb*A copies

After screening 1200 colonies of a cpDNA library, six *psb*A-positive clones were isolated. Five of these clones contained a 3.8 kb *Bam*HI insert and one contained a 2.9 kb *Bam*HI insert. These cloned *Bam*HI fragments correspond to those hybridizing in the Southern blot experiment (Figure 1a-b). The 3.8 kb and the 2.9 kb clones were designated pPC121 and pPC932, and the *psb*A genes they harbour were designated *psb*AI and *psb*AII, respectively. Restriction maps for pPC121 and pPC932 are shown in Figure 2. For the enzymes used the maps are identical, from approximately 700 bp 5′ of *psb*A and down to the 3′-end of the coding sequence (compared to the spinach sequence). Assuming same size for *P. contorta* and spinach *psb*A genes, the *Sac*I site in pPC121 is located immediately downstream of the coding sequence, while pPC932 has a *Nco*I site at the corresponding position. In both ends of the fragment, pPC932

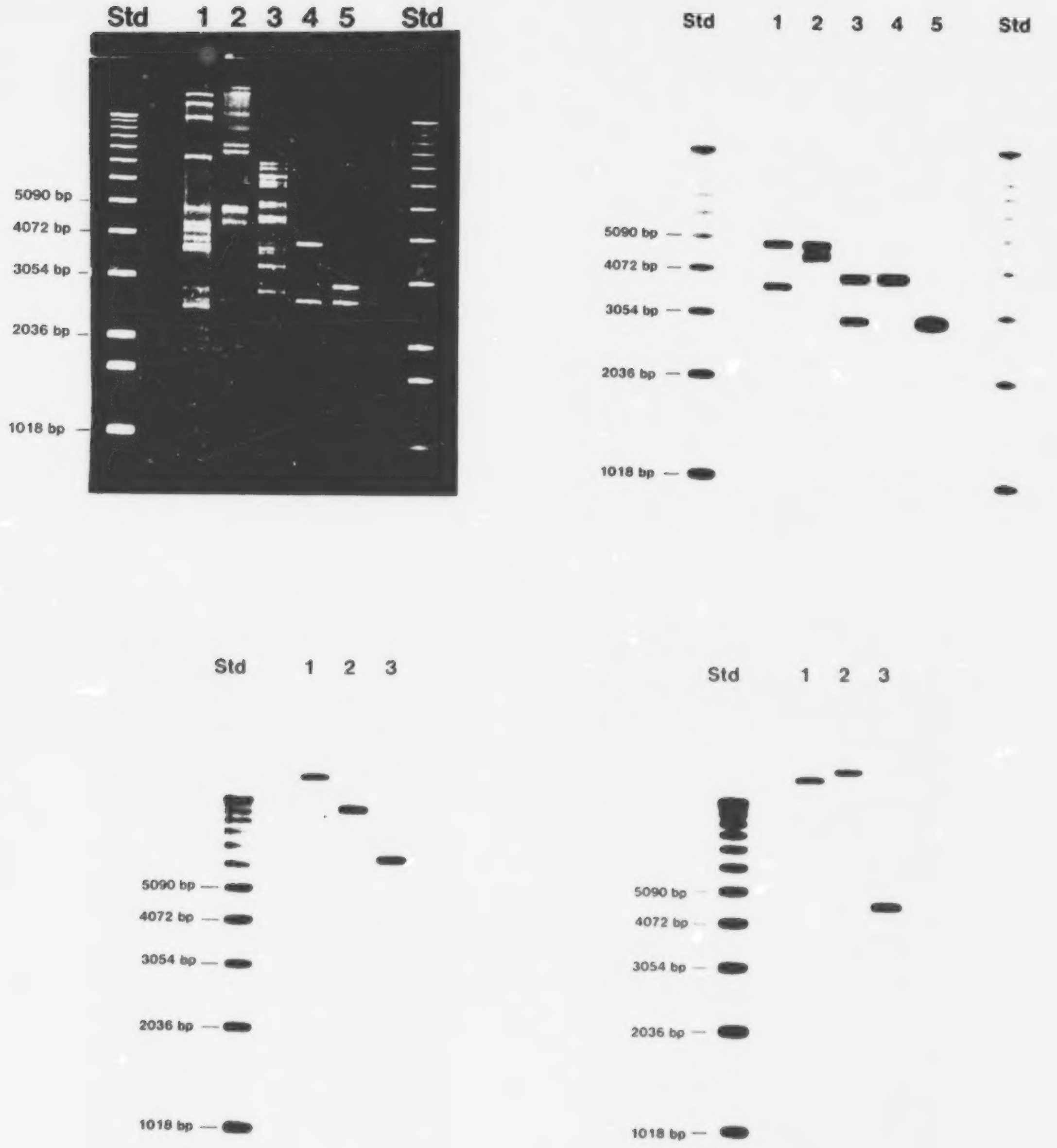
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Figure 1. Agarose gel (a) and Southern blot probed with the spinach *psbA* probe (b) and 16S (c,) and 23S (d) rDNA probes. The cpDNA digests are: *Eco*RI (lane 1), *Sac*I (lane 2), and *Bam*HI (lane 3). Lane 4 and 5: pPC121 and pPC932 respectively, cut with *Bam*HI; the lower of the bands in these lanes are the pUC9 vector.

is truncated by the *Bam*HI sites, as compared to pPC121.

position 280 to 492 of the spinach *psb*A gene (Zurawski et al. 1982), which allowed positioning of the *psb*A genes on the restriction maps (Figure 2). The homology to the spinach *psb*A gene was 87% at the nucleotide level, whereas the deduced amino acid sequences were identical, apart from one leucine to tyrosine substitution at position 117 of the spinach sequence (Figure 3). Although the sequence presented here must be regarded as preliminary because it is based on the sequencing of only one strand, it undoubtedly confirms *psb*A gene identity. Sequencing of both strands is presently underway.

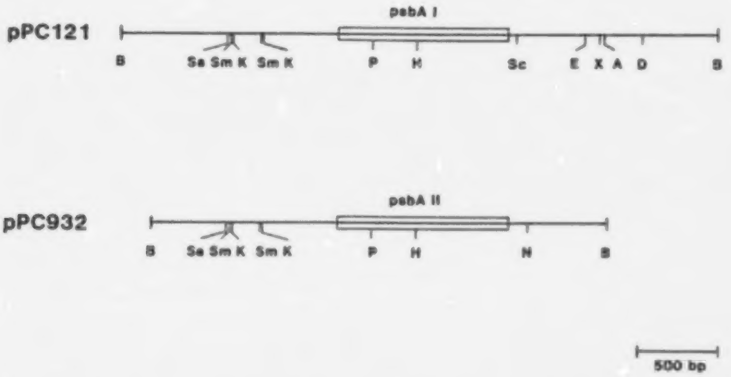
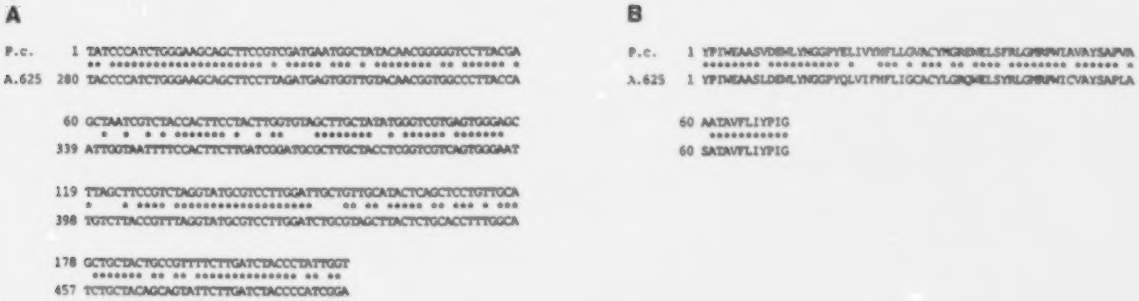


Figure 2. Restriction maps of pPC121 and pPC932. The location indicated for the *psb*A coding region is based on sequence comparison to spinach *psb*A. Recognition sites are indicated for *Ava*I (A), *Bam*HI (B), *Dra*I (D), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Nco*I (N), *Pst*I (P), *Sac*I (Sa), *Sca*I (Sc), *Sma*I (Sm), and *Xho*I (X).

Partial sequencing of the two *psb*A genes

To verify *psb*A gene identity, the 1.0 kb *Sma*I/*Hin*dIII subfragments of pPC121 and pPC932 (Figure 2) were cloned in M13mp18 and partially sequenced from the *Hin*dIII end. The 213 base sequences that could be reliably read from each of the templates were identical and revealed continuous open reading frames (Figure 3). These were found homologuous to


A
P.c.
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B
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Figure 3. Sequence comparison between *P. contorta* (P.c.) and *Spinacia oleracea* (S.o) *psb*A. A: DNA sequence, B: deduced amino acid sequence. Asterisks indicate conserved positions.

Hybridization of rDNA probes to chloroplast DNA

Duplication of the *psb*A gene has also been reported for the green alga *Chlamydomonas reinhardtii* (Erickson et al. 1984) and for the chromophytic alga *Olistodiscus luteus* (Reith and Cattolico 1986). In both cases the duplication is accounted for by the *psb*A location within inverted repeat elements. To examine if *psb*A has a similar location in *P. contorta*, the Southern blot was reprobed with a 16S rDNA specific probe. This probe did not hybridize to the same cpDNA fragments as did the *psb*A probe and, surprisingly, it hybridized to only one cpDNA fragment per digest (Figure 1c). The same result was obtained with a 23S rDNA specific probe (Figure 1d).

Isolation of the 16S containing *Bam*HI fragment

The 6.5 kb *Bam*HI fragment that hybridized to the 16S rDNA probe was isolated from the cpDNA library. Restriction mapping of this fragment, designated pPC28, revealed the 16S gene to be located at one end. From previous Southern blots it was known that the *psb*C and *psb*D genes were on the same fragment and these genes were also positioned on the restriction map (Figure 4).

Discussion

Our knowledge of gymnosperm cp genomes is limited. There have been two reports on isolation and restriction patterns of pine cpDNA (Kondo et al. 1986, White 1986) and, recently, the physical map of the *Ginkgo biloba* L. cp genome was published (Palmer and Stein 1987). It has also been shown that the cpDNA is inherited paternally in conifers (Neale et al. 1986, Szmidt et al. 1987).

In both the green algae *Chlamydomonas reinhardtii* and the chromophytic algae *Olistodiscus luteus*, the *psb*A gene is duplicated. In these organisms the two gene copies are located within the inverted repeats (Erickson et al. 1984, Reith and Cattolico 1986). A similar location in *P. contorta* would have been revealed by hybridization of rDNA probes to the same restriction fragments as the *psb*A probe. In this experiment we unexpectedly found that these probes each hybridized to only one cpDNA fragment per digest. Because these genes are normally duplicated we expected to detect two or more fragments with each of the probes, one from each of the IR-SSC junctions. Thus, *psb*A duplication in *P. contorta* cannot be explained by location within any repeated rDNA structures. The detailed structure of the *psb*A regions suggests duplication of a segment ranging from 700 to 1000 nucleotides upstream of the start codon of the *psb*A coding sequence, to a position very close to the 3′ end of the gene.

It has been demonstrated that the plastid genomes of common bean (Palmer 1983) and the unicellular red alga *Cyanophora paradoxa* (Bohnert and Löffelhardt 1982) exist in two equimolar recombinational forms. The only dif-

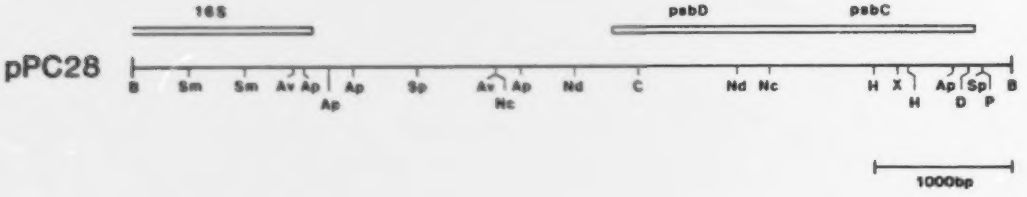

Figure 4. Restriction map of the fragment pPC28. Recognition sites are indicated for *Apa*I (Ap), *Ava*I (Av), *Bam*HI (B), *Cla*I (C), *Dra*I (D), *Hin*dIII (H), *Nco*I (Nc), *Nde*I (ND), *Pst*I (P), *Sma*I (Sm), and *Sph*I (Sp).

ference between these two forms is the orientation of the single copy regions relative to each other. It may be argued that this kind of isomerization could give rise to the *psb*A hybridization pattern we found. However, because there were no inverted repeats in this pine cp genome, this is not likely. Also, we performed a control experiment, where cpDNA from a single *P. contorta* individual was prepared, in order to ascertain that the hybridization patterns observed was not due to populations of different cpDNA genotypes among the seedling material used. This experiment gave the same result as when bulk seedlings were used (not shown). Provided that no other kind of isomerization or heteroplasm exists, we conclude that there is a true duplication of *psb*A.

The sequence homology to spinach *psb*A (Zurawski et al. 1982) is considerable (87% at the nucleotide level), and only one substitution in 71 positions at the deduced protein level was found. This agrees with the well established conservatism of the *psb*A gene. The DNA sequence obtained from pPC121 and pPC932 so far has not revealed any differences.

At present we do not know whether both *psb*A copies are expressed and, hence, the biological function of this gene duplication cannot be assessed. The gene product of *psb*A, the D1 protein, is thought to be an integral part of the photosystem II (PS II) reaction center. It has been demonstrated that D1 protein is a target when excess light damages PS II and that restoration of photosynthetic function is dependent on *de novo* synthesis and replacement of this protein (Kyle et al. 1984). If the synthesis of D1 protein is rate limiting for photosynthesis, the higher gene dosage of the *psb*A gene conferred by the duplication may be of significance.

We have shown in this report that the cp genome of *P. contorta* deviates from the "higher plant consensus" on at least two major points; duplication of the *psb*A gene and absence of one of the rRNA encoding repeats. Among eight other pine species, the *psb*A duplication is found only in *Pinus banksiana* Lamb. (data not shown). This supports both previous taxonomic studies (Critchfield and Little 1966, Mirov 1967) and our own interpretations of restriction analysis of various pine species (Szmidt et al., in prep.), which all recognize *P. contorta* and *P. banksiana* as a distinct taxonomic group. We, therefore, suggest that the *psb*A duplication is the result of a relatively recent rearrangement. In contrast, one inverted repeat segment seems to be lost in all of the pine species examined (data not shown). This is of considerable interest because the cp genome has both the inverted repeats retained in *Ginkgo biloba* (Palmer and Stein 1987), believed to be an ancient gymnosperm. In members of the Fabaceae which also lack one inverted repeat, the *psb*C and *psb*D genes are located close to the 23S gene in the rRNA gene cluster (Rasmussen et al. 1984), while in *P. contorta* they neighbour the 16S rRNA gene. It is evident that the evolutionary events resulting in the loss of one inverted repeat unit in these two groups of plants are unrelated.

The findings presented in this report emphasize the importance of actually examining representatives of different taxonomic groups of plants rather than making extensive generalizations based on results from only distantly related or unrelated species. We believe that our findings also demonstrate the considerable potential for molecular genetics in taxonomic and evolutionary studies.

Acknowledgments

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CHLOROPLAST DNA POLYMORPHISM IN A SYMPATRIC REGION

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Abstract

Chloroplast DNA restriction fragment length polymorphism (RFLP) was studied in 786 individuals from two populations of the *Pinus banksiana* Lamb. - *P. contorta* Dougl. sympatric region. Novel cpDNA variants occurred with a frequency of approximately 4.5% in the sympatric sample, although they have not been detected in the allopatric ranges of either parental species. The sympatric variants might be due to interactions of mutation, selection, and genetic drift in environments of the sympatric region. Alternatively, the novel variants might result from cpDNA sequence alterations induced by interspecific hybridization.

Introduction

Jack pine (*Pinus banksiana* Lamb.) and lodgepole pine (*P. contorta* Dougl.) are known to be polymorphic for a number of morphological, terpene, allozyme, and chloroplast (cp) DNA markers, some of which are useful taxonomically (Critchfield 1985, Wagner et al. 1987). We intensively sampled two populations (Figure 1) in the *P. banksiana* - *P. contorta* sympatric region in order to characterize the cytonuclear genetic structure of putative hybrid populations. In this report we describe the frequencies of cpDNA variants in these two populations and compare the results with previous findings in allopatric populations.

Materials and methods

A large total sample size of 786 individuals was chosen because, if the statistical properties of cytonuclear disequilibria are similar to those of nuclear disequilibria, this sample size allowed reasonable confidence of detecting moderate intensities ($|D'| > 0.2$) of interaction (Brown 1975).

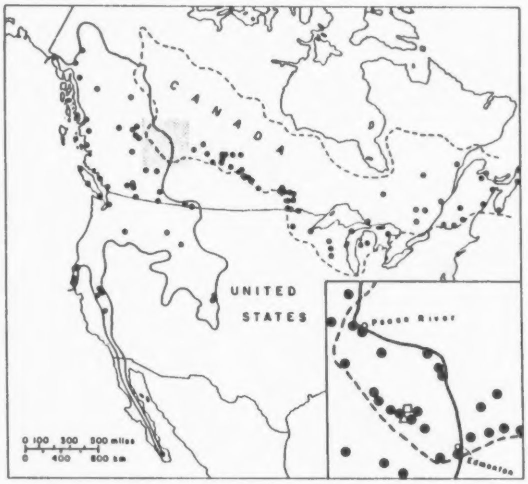

Figure 1. Distributional ranges of *P. banksiana* (broken line) and *P. contorta* (solid line). Filled circles represent populations from which cpDNA phenotypes of 363 individuals have been reported previously (Wagner et al. 1987). Open square (Carson Creek, Alberta) and open triangle (Windfall, Alberta) denote the sympatric populations of this study.

The total sample size was distributed between two populations to allow tests of heterogeneity. Total cellular DNA was prepared from each individual as described previously (Wagner et al. 1987). Pine *Sst*I cpDNA restriction fragments were resolved by agarose gel electrophoresis and Southern hybridization (Southern 1975) to a 9 kilobase pair (kb) *Pst*I fragment from the *Petunia hybrida* chloroplast genome (Palmer et al. 1983). Restriction fragment banding patterns (variants) were identified by the estimated sizes of the variable restriction fragments they contained.

A chi-square test of cpDNA variant frequency heterogeneity among populations was performed after placing each cpDNA variant into one of the following three categories: 1) allopatric lodgepole pine, if a variant had been reported previously in the allopatric range of lodgepole pine; 2) allopatric jack pine, if the variant had been reported previously in the allopatric range of jack pine; or 3) sympatric, if the variant had not been observed previously in either allopatric range (Wagner et al. 1987). This pooling of cells in the contingency table was necessary because of the large number of cells with low frequencies in the complete table.

Results

Significant heterogeneity ($X^2_{(2)} = 9.2$, probability ~ 0.01) existed in the data from the two populations. In Table 1 we present, therefore, the data for each population separately.

Both populations appear to contain cpDNA primarily from *P. contorta*. This predominant influence of *P. contorta* is supported by cone morphology data (D.B.W. and D.R.G., unpublished data).

These data are consistent with previous findings that 4.5/5.0 is the most frequent *P. contorta* cpDNA variant, 4.4/5.0 and 4.7/5.0 are the next most frequent *P. contorta* variants, and 4.8/5.7 is the most frequent *P. banksiana* variant (Wagner et al. 1987). All previously reported cpDNA variants, typical of allopatric *P. banksiana* and *P. contorta*, were also observed in the Carson Creek and Windfall populations. However, this study has revealed the presence, in the region of sympatry, of novel cpDNA variants that have not been identified previously in the allopatric ranges of the two species (Table 1). We have taken a conservative approach and do not consider a cpDNA variant to be novel unless its restriction fragment banding pattern was consistent following at least two independent *Sst*I digestions. Taken together, these novel variants occurred in approximately 4.5% of the individuals sampled in the sympatric region.

Discussion

The statistically significant heterogeneity between the two populations may have resulted from the large sample size. However, this heterogeneity suggests that jack pine cpDNA variation is somewhat more frequent in the Carson Creek population than in the Windfall population (Table 1).

It is necessary to consider the frequencies of the novel cpDNA variants in the sympatric region and in the allopatric ranges of both species in order to calculate the probability that the novel variants occur in the allopatric ranges of the parental species at the same frequency as in the sympatric region. The pooled frequency of novel variants in the sympatric region is 35/786=0.045 (Table 1). The frequency of novel variants in the allopatric ranges of the two species is zero (Wagner et al. 1987). If we employ a conditional Poisson distribution, related to a binomial distribution (Steel and Torrie 1980), the probability that the frequency of novel variants in the sympatric region is the same as in the allopatric ranges is $p = 4.2x10^{-5}$. This extremely low probability indicates that the apparent establishment of novel variants in the sympatric region is not due merely to the large sympatric sample size, but is more likely due to genetic mechanisms that are capable of increasing cpDNA diversity.

There are three possible mechanisms that may account for sympatric cpDNA variants; 1) novel interactions of selection, mutation, and genetic drift in environments of the sympatric region, 2) establishment of cpDNA sequence alterations (e.g. mutations or intramolecular recombination) in homoplasmic individuals due to instability caused by hybridization; and 3) cpDNA sequence alterations (e.g. intermolecular recombination) in heteroplasmic individuals that might arise by maternal leakage of cpDNA (cpDNA is apparently ordinarily inherited paternally in *P. contorta* x *P. banksiana* hybridization events) (Wagner et al. 1987). We cannot reject any of these three alternatives with the present data, but we note that not all of the novel variants can be explained by heteroplasmy alone, because they do not represent simple mixtures of allopatric variants.

Unique nuclear alleles have been reported previously in hybrid zones (Hunt and Selander 1973, Sage and Selander 1979). The explanations offered to explain novel sympatric nuclear alleles have included: a) increased mutation rates in hybrids (corresponding to our alternative no. 2); and b) intragenic recombination (corresponding to our no. 3). Golding and Strobeck (1983)

Table 1. Chloroplast DNA variant frequencies, Carson Creek and Windfall populations.

cpDNA Variant[a]	Frequency		Taxon[b]
	Carson Creek	Windfall	
4.4/5.0	42 (.13)	61 (.14)	L
4.5/5.0	230 (.69)	318 (.71)	L
4.7/5.0	23 (.07)	39 (.09)	L
4.5/4.8	1 (.00)	3 (.01)	L
4.4/5.7	2 (.01)	2 (.00)	J
4.7/5.7	6 (.02)	1 (.00)	J
4.8/5.7	15 (.04)	5 (.01)	J
5.0/5.7	0 (.00)	3 (.01)	J
Several[c]	16 (.05)	19 (.04)	S
Total	335 (1.01)	451 (1.01)	

[a] Variable *Sst*I restriction fragment sizes, kilobase pairs.

[b] Found in allopatric lodgepole pine (L), allopatric jack pine (J), or only in or near sympatric region (S).

[c] Several variants found only in or near sympatric region were pooled due to their rarity.

have provided theoretical evidence that, under certain conditions, intragenic recombination may increase the number of alleles in hybrid zones.

It will be important to determine which mechanisms account for the sympatric cpDNA variants of *P. banksiana - P. contorta*, because they are present in sufficient frequency to be of potential evolutionary consequence. If indeed cpDNA is subject to natural selection, at least one phenotypic effect (fitness) of cpDNA variation would be implicated. Such an effect of cpDNA polymorphism might have relevance in breeding programs. Alternatively, if cpDNA polymorphism has no effect on fitness-related or other phenotypic traits in forest trees, then cpDNA variants would be useful primarily as genetic markers.

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GENETIC POLYMORPHISM FOR NUCLEAR RIBOSOMAL GENE NUMBER IN DOUGLAS-FIR (*PSEUDOTSUGA MENZIESII* [MIRB.] FRANCO.)

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Abstract

Genetic variability for the number of copies of ribosomal genes was studied in 54 trees derived from seed collected throughout the natural range of Douglas-fir *Pseudotsuga menziesii* (Mirb.) Franco. Genetic polymorphism for ribosomal gene copy number was substantial, residing primarily within, rather than among, populations. Copy number tended to increase with latitude, elevation, and longitude. The tendency for copy number of be higher in cold climates concurs with results found for genome size in several other conifers and among herbaceous plant species.

Introduction

There is little consensus over the extent of intraspecific genome size variability in conifers (Teoh and Rees 1976, Dhillon et al. 1977). Nonetheless, in several studies genome size has been found to be greatest in northern populations (Burley 1965, Mergen and Thielges 1967, Miksche 1968, 1971, El-Lakany and Sziklai 1971), paralleling interspecific trends in numerous wild and cultivated plant species (Bennett 1976, Levin and Funderburg 1979). These trends suggest that genome size may affect fitness, and thus be subject to natural or artificial selection for adaptation to cold climates.

Rather than studying genome size, which is difficult to measure and interpret, we chose to study quantitative variation in specific highly repetitive genes and DNA sequences. Even where genome size variation is modest, gene number varies widely. Moreover, individual genes or sequences may have different levels of variability and responds differently to natural selection, depending on their function(s), their location(s) in the genome, degree of iteration, and other factors. We focused on the variability of nuclear ribosomal genes, which exist in tandem arrays or roughly 7200 total copies in Douglas-fir *Pseudotsuga menziesii* [Mirb.] Franco (Long and Dawid 1980).

Materials and Methods

DNA was extracted from foliage of 54 Douglas-firs growing near Corvallis, Oregon. The trees were derived from wind-pollinated seed collected almost throughout the natural range of Douglas-fir, including samples collected in 10 American states, Mexico, and Canada (Table 1). DNA was purified via precipitation with CTAB (Wagner et al. 1987), proteinase K treatment, and banding in cesium chloride. Copy number variation was estimated by a filter hybridization technique called "slot-blotting" (Cullis et al. 1984, Rivin 1986, Rivin et al. 1986). In brief, set quantities of DNA were denatured, neutralized, and loaded into precisely machined wells of a slotted template (see Figure 1). The wells insured that DNA was applied to the filter in a precise area; DNA samples, including Douglas-fir and recombinant ribosomal DNA (rDNA) plasmid standards, were replicated within and among filters and all filters were hybridized in the admixture to 0.3 µg of a nick-translated, radioactive recombinant rDNA plasmid. A plasmid containing one complete repeat unit of soybean rDNA (ca. 7.5 kb insert in pBR325) was used in our experiments (L. Zimmer, Louisiana State Univ., pers. comm., Eckenrode et al. 1985). Each Douglas-fir sample was replicated on two wells per blot on five blots; samples on each blot were independently diluted and quantitated via uv spectrophotometry. Controls were included in filters to assess non-specific binding to the filters and to salmon sperm DNA, which was used as a carrier. Both yielded trivial amounts of hybridization. After hybridization, the filters

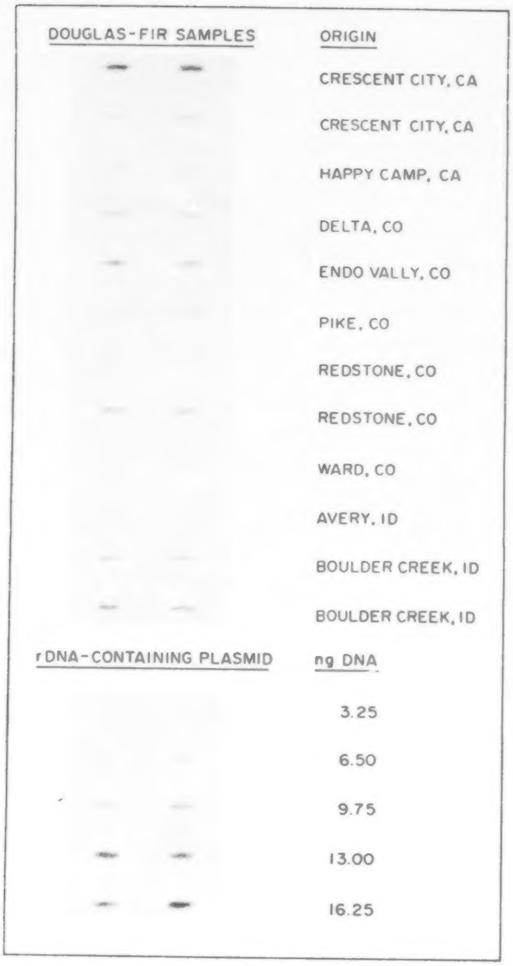
DOUGLAS-FIR SAMPLES
ORIGIN
CRESCENT CITY, CA
CRESCENT CITY, CA
HAPPY CAMP, CA
DELTA, CO
ENDO VALLY, CO
PIKE, CO
REDSTONE, CO
REDSTONE, CO
WARD, CO
AVERY, ID
BOULDER CREEK, ID
BOULDER CREEK, ID
rDNA-CONTAINING PLASMID
ng DNA
3.25
6.50
9.75
13.00
16.25

Figure 1. Autoradiograph of two representative slot blots, the first showing variation among Douglas-fir trees and the second a plasmid dilution series.

were washed at high stringency (0.1XSSC, 65°C) (Maniatis et al. 1982) and exposed to film using intensifying screens. Each well was read twice, giving a total of 20 densitometric readings per Douglas-fir sample over all five blots.

We have expressed copy number in terms of equivalent copy number (ECN), the estimated number of rDNA recombinant plasmids (x 10^{-8}) giving a densitometric signal equal to the mean for that of 100 ng of Douglas-fir DNA. It is intended solely as an estimate of relative, rather than absolute, copy number. All analyses were performed on the product of 3.08 and ECN. Variance among lanes and sets (illustrated in Figure 2) were removed via analysis of variance and are excluded from Table 2 for brevity.

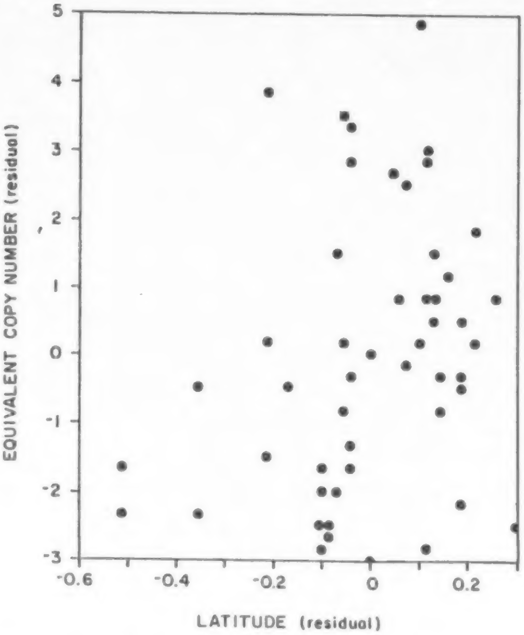

Figure 2. Partial regression plot of ECN and latitude; the values of ECN and latitude are residuals, with a mean of zero and they are corrected for associations with all other variables in the regression model except each other (Table 3). The slope of a linear fit to the points would equal the regression coefficient for latitude in the full model in Table 3.

Table 3. Regression of equivalent copy number (ECN) on geographic variables.

Variable	Degrees of freedom	β(SE)	STD-β	PROB
Intercept	1	-116.306(60.4)	00.00	0.060
Elevation	1	0.008(0.005)	4.18	0.089
Latitude	1	3.126(1.543)	10.72	0.048
Longitude	1	0.995(0.485)	3.42	0.046
El x La	1	$-6.0\times10^{-5}(3.5\times10^{-5})$	-3.47	0.096
Lo x La	1	0.024(0.012)	-12.26	0.054

$R^2$1=0.121; ADJ-R^2=0.027; Sy.x=2.004; CV=17.74%; $\overline{X}$=11.30

Abbreviations: β(SE) = partial regression coefficient (standard error), STD=β = standardized partial regression coefficient, PROB = probability that coefficient is not equal to zero (two-tailed test), ADJ-R^2 = 1-[(1-R^2)(n-1)/(error degrees of freedom)] (SAS 1982), Sy.x = root mean square error, CV = coefficient of variation (%) = (Sy.x/$\bar{x}$)100.

Results

The mean value of ECN was 3.66, and ranged from a low of 2.47 for a tree from Coahuila, Mexico to 12.18 for a tree from near Victoria, British Columbia (#130SE), Canada, nearly a five-fold range (Table 1). The value for the latter tree was unusually high, but our result confirms the measurement in a prior experiment and thus is not due to random measurement error. Excluding this outlier tree from further analyses, the range of ECN was from 2.47 to 5.11. The coastal (var. *menziesii*) and Rocky Mountain (var. *glauca*) varieties of Douglas-fir were essentially coincident in their ECN distributions.

Analysis of variance (Table 2) indicated that trees within populations were by far the major source of variance, accounting for nearly half (44%) of the total variance observed. As a consequence, population variance was calculated to be zero, though the geographic trends observed in regression suggest that weak population differentiation has probably occurred (discussed below).

The regression model had a small R^2 term (12%). It is, however, likely to be considerably inflated, primarily as a result of the large number of dimensions relative to total degrees of freedom in the model. The adjusted R^2 value of 3% is probably a more realistic estimate of the degree to which variance in ECN is explained by geography. The regression coefficients indicated that ECN tends to be higher in colder climates, as evidenced by positive regression coefficients for latitude and elevation (Table 2B). These trends were primarily a result of trees from low latitudes and elevations having low ECNs; at high latitudes and elevations, despite great variance in ECN, the variables appeared unrelated (e.g., Figure 2). Combinations of high elevation and latitude, and high longitude and latitude, had ECNs that were lower than expected based on the main effect trends. This is shown by the negative interaction coefficients and inspection of plots (not presented), similar to that of Figure 2. The positive coefficient for longitude indicated that ECN tended to increase toward the west.

Discussion

Ribosomal DNA copy number showed large genetic polymorphism within populations, but small genetic differentiation among varieties or populations. Geographic trends are nonetheless evident which, despite modest levels of statistical significance, concur with those found for genome size in several conifer species and among numerous angiosperm species: larger genomes and copy numbers occur in cooler climates.

Two hypotheses for these trends seem tenable. First, larger ribosomal and protein-coding gene dosages may allow more rapid protein synthesis, and thus more rapid cell expansion in cool, moist conditions (Grime and Mowforth 1982). If true, rDNA copy number may be a useful screening tool for breeding programs aimed at enhancing yield in cold environments. Second, if the rate (or cost) of DNA synthesis becomes progressively less limiting to growth as temperatures decrease (i.e., other metabolic limitations grow in relative importance), then selection against the hypothesized "natural" tendency for genome size to increase may be relaxed (e.g., Loomis and Gilpin 1986). If this is so, we might expect rDNA dosage to increase more rapidly than the bulk of the genome because long spans of tandem repeats may promote unequal crossovers and gene amplification. Thus, larger genomes and gene dosages may serve no adaptive function; they may simply be a result, rather than an adaptive mechanism, of evolution in cool environments.

The latitudinal trends we observed fit best with this latter hypothesis (Figure 2). At low latitudes gene number seems constrained downward. At middle to high latitudes, however, gene number varies widely, overlapping that of the southern latitudes. Its weak relationship to geography at high latitudes is difficult to reconcile with the hypothesis that rDNA copy number serves an important adaptive function. However, this study was designed as a genetic survey and not as a test of adaptation.

There are numerous reasons why only a weak relationship between rDNA copy number and geography might have been observed, even were copy number *per se* to be of selective value. For example, environmental variation is poorly

Table 1. Source of plant material and their mean equivalent copy number of ribosomal genes (ECN)

State	Locality	Tree ID	Elevation (meters)	Latitude (degrees N)	Longitude (degrees W)	ECN
AZ	Flagstaff	1033	2408	35.19	111.61	4.64
AZ	Flagstaff	1033B	2408	35.19	111.61	2.75
AZ	Signal Mt.	1027A	2287	33.50	110.75	3.74
AZ	Signal Mt.	1027B	2287	33.50	110.75	3.85
AZ	Stafford	582	2591	32.84	109.68	4.21
AZ	Tucson	266	2439	32.50	110.80	3.64
BC	Quesnel	124NE	475	52.98	122.50	3.43
BC	Quesnel	124NW	475	52.98	122.50	3.43
BC	Salmon Arm	126N	533	50.75	119.10	3.44
BC	Salmon Arm	126S	533	50.75	119.10	3.86
BC	Victoria	34NE	0	48.60	123.50	4.56
BC	Victoria	130SE	0	48.80	123.50	12.18
CA	Crescent City	607SE	0	41.75	124.20	4.55
CA	Crescent City	607SW	0	41.75	124.20	3.18
CA	Happy Camp	1117	332	41.80	123.40	2.88
CO	Delta	567	2530	38.70	108.20	3.76
CO	Endovalley	875	2591	40.30	105.70	4.18
CO	Pike Nat. For.	910E	2348	38.80	105.10	3.10
CO	Redstone	385NW	2183	39.20	107.20	4.73
CO	Redstone	385SE	2183	39.20	107.20	3.53
CO	Ward	891	2866	40.20	105.50	3.19
ID	Avery	177NE	915	47.30	115.75	3.68
ID	Boulder Creek	202NE	1296	48.75	116.50	3.83
ID	Boulder Creek	202SE	1296	48.75	116.50	5.11
ID	Cedars	164	1158	46.60	115.75	3.49
ID	Emida	180	610	47.20	116.60	3.63
ID	Emida	189	610	47.20	116.60	3.86
ID	Shoshone	724	1219	47.50	115.75	3.03
ID	Stites	49	402	46.10	116.00	2.85
MT	Clacier Pk.	775	1037	48.75	113.70	3.62
MT	Troy	753	1037	48.50	115.80	3.38
MT	White Pine	735	884	47.75	115.50	4.68
MT	White Pine	739	884	47.75	115.50	4.06
MX	Coahuila	1105A	2460	25.25	100.50	2.47
MX	Coahuila	1105B	2460	25.25	100.50	2.69
NM	Cloudcroft	281	2641	33.10	105.75	4.38
NM	Monjeau Lookout	288	3049	32.40	105.00	3.78
NM	Monjeau Lookout	288N	3049	32.40	105.00	2.85
NM	Mt. Taylor	930	2668	33.25	108.30	3.98
NM	Mt. Taylor	946	2866	35.25	108.30	4.14
NM	Reserve	303NE	2987	33.70	108.70	3.94

continued

Table 1. continued

State	Locality	Tree ID	Elevation (metres)	Latitude (degrees N)	Longitude (degrees W)	ECN
OR	Blodgett	41	193	44.60	123.50	4.84
OR	Santiam Junction	630NE	145	44.43	121.94	3.68
OR	Santiam Junction	630NW	1145	44.43	121.94	3.11
UT	Kamas	412W	589	40.60	111.30	3.53
UT	Scipio	421E	2439	39.30	112.20	2.83
UT	Scipio	421W	2439	39.30	112.20	3.12
WA	Forks	458NE	93	47.90	124.50	3.15
WA	Issaquah	475E	23	47.50	122.10	3.44
WA	Issaquah	475W	23	47.50	122.10	3.63
WA	Lake Quenault	473NW	67	47.40	123.75	3.76
WA	Lake Quenault	473SW	67	47.40	123.75	2.75
WA	Waterville	509SW	799	47.60	120.10	3.68
WY	Mt. View	557	2439	42.90	106.20	2.88

Abbreviations: ECN = [Number of ribosomal gene containing pGmrl plasmids giving an autoradiographic density equivalent to 100 ng of Douglas-fir DNA] x 10^{-8}, AZ = Arizona, BC = British Columbia, CA = California, CO = Colorado, ID = Idaho, MT = Montana, MX = Mexico, NM = New Mexico, OR = Oregon, UT = Utah, WA = Washington, WY = Wyoming, Mt. = Mountain, Pk. = Park.

Table 2. Analysis of variance of equivalent copy number (ECN)

Source	Degrees of freedom	Mean squares	Variance	Variance %	Expected Composition of Mean Square
Blot = B	20	57.75[a]	1.11	12.9***	$\sigma^2_r + 4.0\,\sigma^2_{btp} + 6.5\,\sigma^2_{bp} + 42.4\,\sigma^2_{bs}$
Population = P	32	81.61	-0.12	0	$\sigma^2_r + 4.0\,\sigma^2_{btp} + 5.6\,\sigma^2_{bp} + 19.9\,\sigma^2_{tp} + 28.0\,\sigma^2_p$
Trees (P) = TP	16	79.90[b]	3.78	44.0***	$\sigma^2_r + 4.0\,\sigma^2_{btp} + 19.9\,\sigma^2_{tp}$
Blot x P = BP	128	9.58[b]	0.90	10.5**	$\sigma^2_r + 4.0\,\sigma^2_{btp} + 5.6\,\sigma^2_{bp}$
Blot x TP = BTP	63	4.54	0.59	6.8	$\sigma^2_r + 4.0\,\sigma^2_{btp}$
Residual = R	795	2.18	2.18	25.5	σ^2_r
Corrected Total	1059	8.26	8.40	99.7	–

[a] Tested against BP mean square after adjustment for difference in coefficient of bps variance terms.

[b] Tested against BTP mean square.

indexed by latitude, longitude, and elevation; transcriptional activity may differ among gene copies; somatic variation and selection during growth in Corvallis may have obscured some population differences; and, genome size variation may exist, possibly altering total ribosomal gene dosage without affecting its proportion in the genome. Analyses of the adaptive value of ribosomal gene number variation would benefit from a study of simple environmental gradients, from quantification of the number of transcriptionally-active ribosomal gene copies, and from analysis of the consequences of rDNA dosage polymorphism within populations, because this is where the great bulk of genetic variability resides.

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STABILITY OF HIGHLY REPEATED SEQUENCES IN THE DNA OF EMBRYOGENIC CULTURES OF YELLOW POPLAR

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Abstract

Embryogenic lines of yellow poplar (*Liriodendron tulipifera* L.) were employed in a study to characterize genome organization, isolate highly repeated DNA sequences, and test for among- and within-clone variation in yellow poplar DNA. We used restriction fragment polymorphisms and highly-repeated DNA probes. The melting temperature of yellow poplar DNA was determined to be 85°C. From reassociation kinetics, it was estimated that 22% of the DNA sequences in the genome were highly repeated and 68% were single-copy sequences. The copy number of highly repeated sequences was estimated to be 1.6×10^6. Restriction fragment polymorphisms could be detected, among half-sib embryogenic suspension culture lines and among plantlets regenerated from different lines, using a probe containing highly repeated DNA. However, no such polymorphisms could be detected among sublines of a given embryogenic line or among plantlets regenerated from a given embryogenic line.

Introduction

Somaclonal variation has been defined as a genetic variation among plants regenerated from somatic cells using tissue culture (Evans and Sharp 1986). It has been debated whether this variation is the result of genetic differences preexisting in the somatic cells of the explant or is induced by components of the culture medium, although it appears that most of the variation arises during cell culture (Scowcroft et al. 1983). There are numerous reports of somaclonal variation in herbaceous species, including several important crops (Scowcroft et al. 1983). However, there are few published reports of somaclonal variation among woody species (Ahuja 1987). Lester and Berbee (1977) found a wide range of variation in height, branch number, leaf traits, and chromosome number in plants regenerated from callus of *Populus*. Although gross chromosomal changes such as aneuploidy and polyploidy have often been observed in plant tissue cultures, variant phenotypes have often not been associated with karyotype changes. Instead, there has been evidence of other, more subtle mechanisms, including chromosome rearrangements and single gene mutations (Scowcroft et al. 1983, Evans and Sharp 1983).

Other genetic changes that could cause phenotypic variation include the action of transposable elements, mitotic crossing over, and gene amplification and depletion (Scowcroft et al. 1983). In this last area of gene amplification and depletion, Cullis and Cleary (1986) showed that the genome of flax cells in culture and of plants regenerated from callus can differ from the genome of the plant from which they were derived, displaying both increases and decreases in the proportions of certain highly repeated DNA sequences. We decided to apply a crude variant of Cullis and Cleary's (1986) approach to our embryogenic yellow poplar (*Liriodendron tulipifera* L.) suspension lines and regenerated plantlets. The primary objective of the study was to employ highly repeated DNA probes and restriction fragment polymorphisms as a quick screen for DNA variation among and within our yellow poplar lines. Other objectives, which were necessary prerequisites to the use of these highly repeated DNA sequences, were to characterize the genome of yellow poplar as to melting temperature (T_m), genome size, and proportions of highly repeated DNA and single-copy sequences, and to isolate the highly repeated sequences to be used as probes.

Materials and methods

Cell subline establishment

The embryogenic yellow poplar lines (Merkle and Sommer 1986) designated as lines 28, 35, 39, and 65 were employed in this study. Each line was grown in suspension culture until the late stationary phase in order to subject it to a "stress environment". The cells were then harvested and plated using a modified double-layer technique (Miller 1972) in order to obtain single uniform cell clumps. After two weeks, cell clumps were selected and placed in individual wells of tissue culture "cluster" plates in liquid medium to create several sublines for each of the four embryogenic parent lines. Later, these sublines were transferred to 125 mL flasks and grown in suspension culture.

Genome characterization

Yellow poplar DNA was isolated from suspension-cultured cells using a modified Murray and Thompson (1980) technique. The melting temperature (T_m), which is the midpoint of the temperature range over which the DNA is denatured, was determined using the following procedure: purified DNA was dissolved in 0.12 M phosphate buffer, transferred to a stoppered cuvette, and incubated in a water bath at 45°C. Its optical density at 260 nm (OD_{260}) was read at this temperature and, using the water bath, the temperature was gradually increased. The optical density was read at 10° intervals until a maximum plateau was reached. Using this procedure, T_m was estimated to be 85°C.

Other characteristics of the yellow poplar genome, including genome size and proportions of highly repeated and single-copy DNA sequences, were determined using C_0t curve analysis. C_0t is the product of DNA concentration in moles (C_0) and the time of incubation (t). To construct a C_0t curve, C_0t is plotted on the X-axis and the percentage of DNA that has reassociated ($1- C_0/C_0$) is plotted on the Y-axis. Pure DNA was dissolved in 0.12 M phosphate buffer and sheared to 0.3-100 kilobase (kb) pairs. The sheared DNA fragments were denatured at 92°C and the OD_{260} was read. The sample was then incubated at 60°C and passed through a hydroxyapatite column which absorbs double-stranded DNA. The OD_{260} of the eluted single-stranded DNA was read to determine the reassociation fraction. The procedure (incubation, hydroxyapatite column, OD_{260} reading) was repeated until an OD_{260} value was obtained at complete reassociation. The C_0t value was calculated for each OD_{260} reading and the C_0t curve plotted. From the C_0t curve, it was determined that approximately 22% of the sequences were highly repeated DNA, with a copy number of 1.6×10^6. Using the unique sequence fraction, which comprised 68% of the sequences, the yellow poplar genome size was estimated to be 3×10^9 base pairs.

Isolation of highly-repeated DNA

Highly repeated DNA sequences were isolated using a method derived from that used by Ohtsubo and Ohtsubo (1976). Total DNA was partially digested using the restriction endonuclease *Bam*HI to obtain 0.3-100 kb DNA fragments. The digestion was carried out at 37°C for 1 h. The DNA was then completely denatured using 1 M NaOH, and incubated to a C_0t value of 7×10^{-4} in a 60°C water bath. From the C_0t curve, we expected that only highly repeated sequences would reassociate at this C_0t value. The reassociation was quickly terminated by quenching in ice. Then the DNA was treated with S1 nuclease at 37°C for 30 min to digest unreassociated single-stranded DNA.

Southern hybridization

The pure highly repeated DNA fragments were labelled with [α-^{32}P] dGTP by *in vitro* nick translation (Maniatis et al. 1982). Total DNA of each suspension-cultured subline was completely digested with *Bam*HI and the resulting fragments separated by electrophoresis on a 0.7% agarose gel. The DNA fragments were denatured on the gel and the resulting single strands transferred to MSI paper by Southern transfer (Southern 1975). The DNA on the MSI paper was hybridized with the radioactive probe prepared above for 40 h at 68°C. Autoradiography was used to visualize hybridization.

Total DNA was also isolated from leaves of individual plantlets regenerated from the four embryogenic yellow poplar lines (Merkle and Sommer 1986). The DNA from each plantlet was partially digested with *Bam*HI, separated by electrophoresis, transferred to MSI paper, and hybridized with the highly repeated DNA probe as described above; autoradiographs of the hybridizations were prepared.

Results and discussion

Phenotypes of sublines of two of the embryogenic yellow poplar lines (65 and 28) varied with respect to morphology, colour, growth rate, and production of secondary metabolites. Upon transfer to fresh medium in 125 mL flasks, sublines of line 35 reverted to their normal cell clump type quickly, while sublines of lines 65, 28, and 39 required more time to revert to their normal phenotypes.

Plantlets regenerated from different embryogenic lines were phenotypically distinguishable with respect to growth rate, internode length, and leaf shape. However, no phenotypic variants could be identified among plantlets within the same clone.

Banding patterns obtained from the Southern hybridizations showed significant differences among embryogenic suspension culture lines, indicating that restriction fragment polymorphisms exist among these lines that can be detected using the highly repeated DNA probe. Similarly, the banding patterns obtained from DNA isolated from individual plantlets regenerated from these lines displayed evidence of restriction fragment polymorphisms among lines. However, DNA isolated from sublines of a given embryogenic suspension culture line showed no discernible variation--even among sublines with variant phenotypes. Comparison of banding patterns among plantlets derived from the same clonal line also failed to display any variation in restriction fragments, using the highly repeated DNA probe.

Our failure to detect restriction fragment polymorphisms among sublines or regenerated plantlets of a given yellow poplar clonal line could be due to a number of reasons. It is possible that the probe we used, consisting of all the highly repeated DNA sequences from the yellow poplar genome, was too crude for differences to show up when the probe was hybridized to the genomic DNA. If this is the case, the next logical step would be to clone individual highly repeated DNA fragments in order to obtain pure highly repeated DNA clones to use as probes.

However, the fact that we did detect variation in the restriction fragment patterns between clonal lines using the crude probe showed that this probe can be used as a molecular marker. It should be noted here that the four clonal lines in the study were derived from zygotic embryos from open-pollinated seed of the same mother tree. Thus the highly repeated DNA probe was sensitive enough to detect differences among half-sib lines.

It is also possible that the genome of this particular species, which is believed to be quite old evolutionarily, is simply very stable in culture. As noted earlier, plantlets of a given clone do appear to be uniform in morphological characters. This remains true even for plantlets regenerated from cultures that are over three years old. Some have argued that somatic embryogenic systems by their very nature avoid somaclonal variation, but Larkin et al. (1983) argue against this theory and cite several examples supporting their position. We have yet to use the highly repeated DNA probe to search for restriction fragment polymorphisms among yellow poplar protoclones (Merkle and Sommer 1987) derived from the same parental line. It is possible that protoplast isolation and culture are associated with higher frequencies of somaclonal variation although, again, Larkin et al. (1983) concluded that the limited evidence available suggests that the frequency of somaclonal variants will not be any higher with protoplast-derived clones than with nonprotoplast-derived ones.

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CHLOROPLAST ULTRASTRUCTURE AND GENE EXPRESSION IN EMBRYOGENIC CONIFER CALLUS.

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Abstract

Embryogenic conifer calli are characteristically white or translucent in appearance even when grown in light. Ultrastructural examination of the embryogenic Norway spruce *Picea abies* (L.) Karst. callus has revealed that somatic embryos contain plastids resembling proplastids found in zygotic embryos. Mature chloroplasts, containing grana, are found in non-embryogenic calli which are green when grown under identical conditions. Proplastids are also found in embryogenic calli of other conifer species. The morphology of the plastids change as the somatic embryos develop. Soluble proteins extracted from both callus types were compared by SDS-PAGE. Differences in proteins extracted from the two tissue types were observed, some of which may relate to chloroplast development and ultrastructure.

Introduction

A feature of embryogenic Norway spruce callus is its translucent appearance, even when grown in light (Hakman et al. 1985). Non-embryogenic Norway spruce calli are characteristically green when grown under these conditions. Embryogenic calli of European larch *Larix decidua* Mill., Douglas-fir *Pseudotsuga menziesii* (Mirb.) Franco, white pine *Pinus strobus* L., loblolly pine *P. taeda* L., and pond pine *P. serotina* Michx. also exhibit this characteristic white or translucent phenotype (Gupta and Durzan 1987, Hakman and Fowke 1987, Nagmani and Bonga 1985, Wann et al. 1987a). In addition to biochemical studies of the two callus types (Wann et al. 1987b), ultrastructural and molecular examinations of the cells are being carried out to better characterize embryogenic conifer calli. We wished to determine if the lack of green coloration of the embryogenic calli was due to a deficiency of chlorophyll within chloroplasts, the absence of chloroplasts, or the presence of an alternate form of plastid such as a proplastid which is typically found in early zygotic embryos or meristems.

Mature chloroplasts arise from progenitor plastids in meristems and embryos via a defined developmental pathway, progressing from eoplasts, amyloplasts, and amoeboid plastids (termed proplastids) to mature chloroplasts. Although the terminology and order of progression through the proplastid forms is subject to some variation in the literature, the general scheme outlined above appears to be well accepted (Thomson and Whately 1980). My studies of the ultrastructure of cultured and intact tissues have concentrated on the morphology of plastids. To date, chloroplasts (plastids) in all of the above mentioned conifer calli (green and white calli) have been examined. Zygotic and somatic embryos of several species, too, have been investigated. Soluble proteins were separated by SDS-PAGE and visualized by silver staining, and relative levels of the mRNA encoding the small subunit of ribulose 1,5-bisphosphate carboxylase (Rubisco) were compared to determine differences in the expression of a protein found in the chloroplasts of embryogenic and non-embryogenic calli. Embryogenic conifer calli contain chloroplasts (plastids) that differ from mature chloroplasts not only morphologically, as illustrated by microscopic examinations, but also biochemically, as suggested by SDS-PAGE and other gene expression studies.

Materials and Methods

Cultures of embryogenic and non-embryogenic conifer callus are available as part of the ongoing work of the tissue culture project at The Institute of Paper Chemistry. Cultured tissues included Norway spruce *Picea abies* (L.) Karst., white pine *Picea glauca* (Moench) Voss, European larch, loblolly pine, white pine, Douglas-fir, and pond pine that were generally initiated from embryos as previously described

(Hakman and Fowke 1987, Hakman et al. 1985, Wann et al. 1987b).

Samples were prepared for transmission electron microscopy (TEM) as follows: Tissue was fixed in 3% glutaraldehyde in 0.05M sodium cacodylate buffer, pH 7.0 (also containing .05% calcium chloride and 0.1 M glucose) for 2-3 h, washed briefly with buffer, and postfixed in 1% OsO_4 in the same buffer for 2-3 h at room temperature. After a brief wash, samples were slowly dehydrated in ethanol at 4°C, followed by slow infiltration with SPURR at room temperature and embedding by polymerization at 70°C for 6 h. The tissues were sectioned at 90 nm and stained with uranyl acetate (5% in 50% (v/v) ethanol) followed by lead citrate (0.5%, pH 12). To insure penetration/infiltration of fixative into seedling cotyledon sections and zygotic embryos, the tissues were subjected to several cycles of vacuum/atmospheric pressure while in the fixative.

Cotyledons were collected from seedlings germinated under normal greenhouse conditions (light grown) or in complete darkness (dark grown; all handling and sample collection was performed under a dim green safelight) 2-3 weeks after the seeds were sown. Total chlorophyll was estimated by determining the A_{652} of an 80% acetone extract of the tissues. The optical density was then used to calculate µg chlorophyll. Soluble proteins were extracted from embryogenic or non-embryogenic calli by homogenizing the tissue in cold 50 mM HEPES (pH=7.5), 1 mM PMSF. After centrifuging for 5 min in an Eppendorf microfuge, protein in the supernatant was quantified by the Bradford dye binding assay and loaded onto standard 12% or 7.5-15% gradient SDS-polyacrylamide gels. Equal amounts of protein (2-3 µg) were loaded onto each lane and visualized in the gel using a modified silver stain technique.

Results and Discussion

When total chlorophyll levels were estimated in acetone extracts of the callus tissues, the results obtained were not surprising and simply confirmed, on a quantitative basis, visual observations that had been described earlier (Figure 1). The chloroplasts of light grown conifers are typical of those found in most higher plants. The organelles, usually 2 to 10 microns in length, contain internal thylakoid membranes organized into grana, and occasional starch grains and protein bodies (Figure 2a). The or-

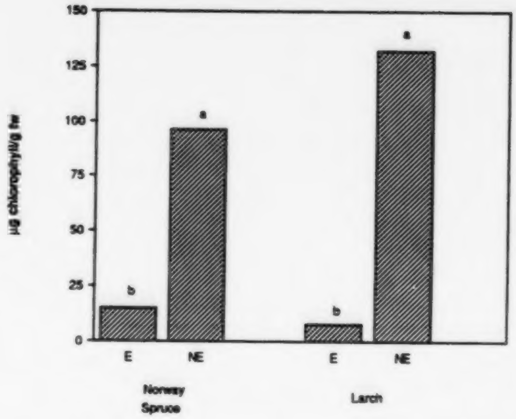

Figure 1. Chlorophyll levels in embryogenic (E) and non-embryogenic (NE) conifer calli.

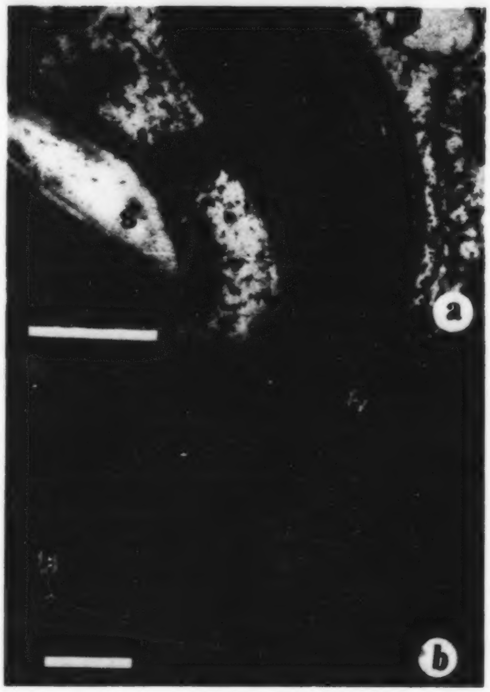

Figure 2. Typical chloroplasts found in green conifer tissue (taken from Douglas-fir nonembryogenic callus) (a) and a chloroplast present in dark-grown Norway spruce seedling cotyledon (b). Legend: bar = 1 micron, G = grana, M = mitochondrion, N = nucleus, P = proplastid, Pb = prolamellar body, S = starch grain.

ganization of the thylakoid membranes is dependant upon the age and physiological state of the tissue (Figure 2b).

The plastids in the embryogenic spruce callus exhibit a different ultrastructure. The plastids, which appear more darkly stained (more electron dense) than mitochondria in the photographs, lack the internal organization of a fully developed chloroplast. At higher magnification some mottling is evident, which may reflect the presence of thylakoid membranes which are in a disorganized state (Figures 3a,b). Some of these plastids contain small starch grains, although they are not nearly as large as the starch grains present in mature chloroplasts in leaf or cotyledon tissue. The plastids present in the green, non-embryogenic spruce tissues appear to be similar to a typical chloroplast (Figures 3c,d). Thylakoid membranes, some organized into grana, are found in chloroplasts. Large starch grains are also prominent in some of the chloroplasts.

Zygotic embryos excised from immature loblolly pine seeds were also examined by TEM. As

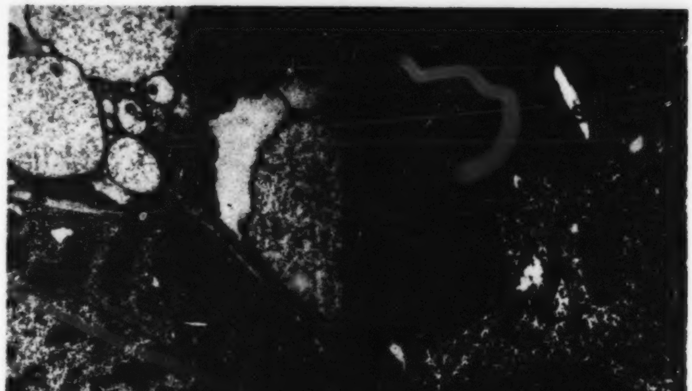

Figure 3. Plastids present in embryogenic (a,b) and nonembryogenic Norway spruce calli (c,d).

shown in Figure 4, plastids lacking organized thylakoid membranes were abundant. Although a significant number contain starch grains, they resemble the plastids observed in embryogenic spruce calli.

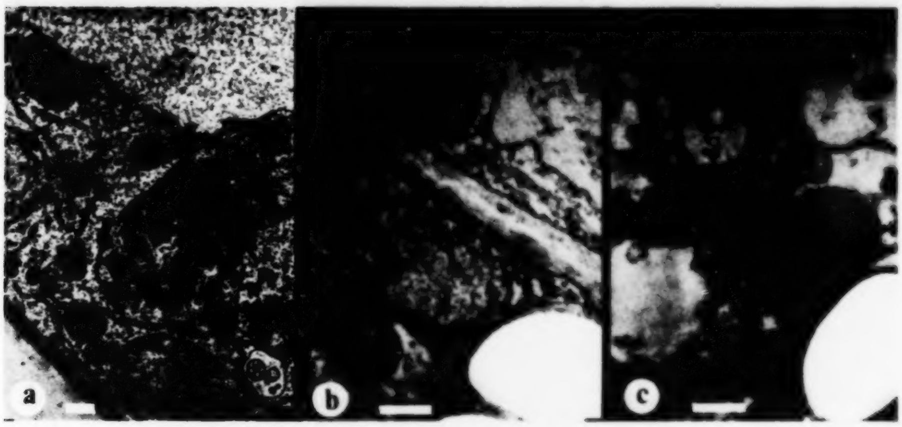

Figure 4. Plastids present in white pine (a), pond pine (b), and loblolly pine (c) embryogenic calli.

In order to determine if the plastid morphology observed in somatic embryos was unique to Norway spruce, embryogenic calli of larch, loblolly pine, pond pine, white pine, and Douglas-fir were also examined. Embryogenic calli of these conifer species contain plastids that closely resemble those in the embryogenic Norway spruce calli (Figures 5,6). Irregularly shaped plastids lacking a significant degree of thylakoid membrane organization or grana formation were noted. Non-embryogenic calli of these species generally contained well developed chloroplasts, very similar to those in non-embryogenic Norway spruce calli. The plastids observed in somatic embryos of carrot, a model system in which development is well characterized, exhibit the same morphology as both embryogenic conifer calli (data not shown). In most higher plants, including conifers, several aspects of chloroplast development are regulated by light. Progenitors of chloroplasts, the proplastids, are stimulated by light during germination to form mature chloroplasts. If seeds are germinated in the dark or if other tissues are grown in darkness, chloroplast development may be altered such that etioplasts are formed (Figure 2b). Etioplasts are colourless, hence the tissues do not appear green, and contain thylakoid membranes that are not organized in grana. A prominent prolamellar body is normally present, which may represent a site of temporary storage of the thylakoid membrane components. Upon further development, such as that stimulated by light, thylakoid membranes are formed and appear to radiate from the prolamellar bodies to form grana. It is obvious that chloroplasts in dark-grown, pale colored Norway spruce and other conifer seedlings (data not shown) are different from those in pale or translucent embryogenic calli. Clearly, proplastids in the embryogenic calli are not due to a simple lesion in the light induced development of the mature chloroplast.

The plastids observed in our tissues closely resemble proplastids found in developing zygotic embryos. In perhaps the most thorough ultrastructural examination of zygotic embryogenesis reported to date the embryonic development of *Capsella* (shepherd's purse) was followed from the egg, 2-cell, 3-cell, heart, and torpedo stages through to the mature embryo (Schulz and Jensen 1968a, 1968b). In the above reports, proplastids, similar to those observed in our study, were found in the one-cell zygote through to the heart stage embryos. Zygotic embryos of black pine (*Pinus nigra* Arnold) and jack pine (*Pinus banksiana* Lamb.) have been examined at the ultrastructural level (Durzan et al.

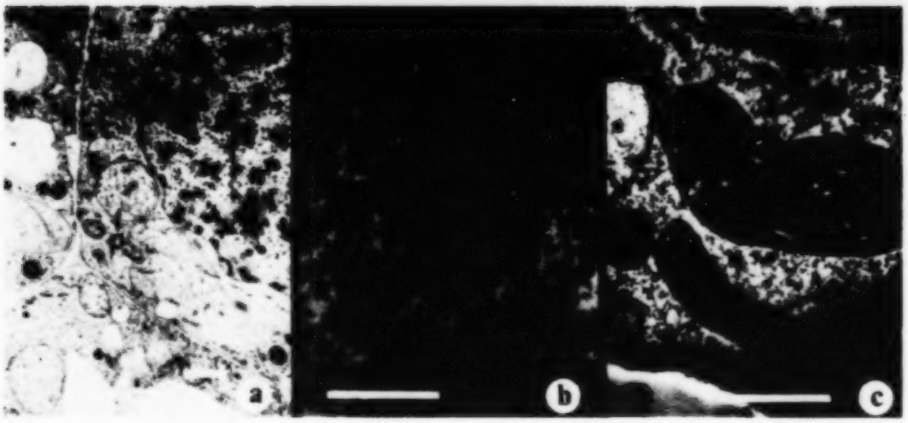

Figure 5. Plastids found in immature loblolly pine embryos.

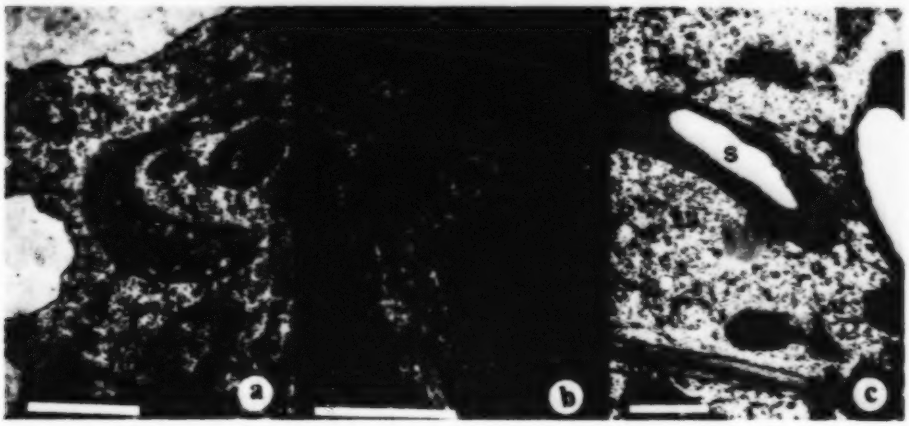

Figure 6. Plastids present in embryogenic (a,b) and nonembryogenic (c) larch calli.

1971, Nikolik and Bogdanovik 1972); the authors stated that proplastids were difficult to recognize before germination or simply identified the plastids as proplastids and did not elaborate on their presence or significance. However, proplastids similar to those noted in our embryogenic calli and zygotic embryos are evident in micrographs in both of these reports.

Relatively few reports describing ultrastructural studies of cultured plant tissues exist in the literature, and only a handful of these include observations of plastid or chloroplast morphology. Chloroplast differentiation has been observed in cultured tobacco calli and carrot calli; in these the morphology of the plastids were noted to change from chromoplasts and leukoplasts to fully developed chloroplasts (Kumar et al. 1984, Seyer et al. 1975). However, tissues examined in these studies were not from somatic embryos but, instead, included proliferating callus. Some plastids in these reports did appear to be similar to those found in our embryogenic callus. Recently, Profumo et al. (1987) described ultrastructural differences between embryogenic and non-embryogenic callus of *Aesculus*. Undifferentiated plastids or plastids devoid of an inner membrane system were noted in their embryogenic calli.

The proteins separated and visualized by SDS-PAGE showed clear differences between embryogenic and non-embryogenic calli, with the exception of white spruce (Figure 7). White spruce also failed to exhibit other biochemical differences which are used to characterize the two callus types (unpublished data, Institute of Paper Chemistry). The most obvious difference observed on the protein gels concerns the presence of a prominent protein of approximately 18-20 kd. This protein is interesting not only because it appears to be abundant in non-embryogenic tissues, but also because it appears to be the same size in all the species examined, excepting white spruce. The abundance and size of the protein suggests that it could be an unprocessed form of the small subunit (ssu) of Rubisco. The ssu of Rubisco, a highly conserved protein encoded in nuclear DNA, is typically processed upon import into the chloroplast to remove a ~5 kd transit peptide to yield a mature protein of ~15 kd. The abundant protein in our preparations is about 5 kd larger than the mature Rubisco ssu (as identified by co-electrophoresis with a major band present in extracts of light grown cotyledons of larch seedlings), suggesting that the abundant protein may be the ssu precursor. It must be noted, however, that the ssu precursor rarely, if ever, accumulates *in vivo*. Preliminary RNA dot blot data (data not shown), generated using a ssu-Rubisco probe isolated from a larch cDNA library, suggests that Rubisco ssu transcript levels are higher in the non-embryogenic callus as compared to the embryogenic callus. This finding would support the hypothesis that the abundant band in the non-embryogenic protein preparations is Rubisco ssu, but the RNA dot blot test and a Northern blot test need to be repeated for confirmation.

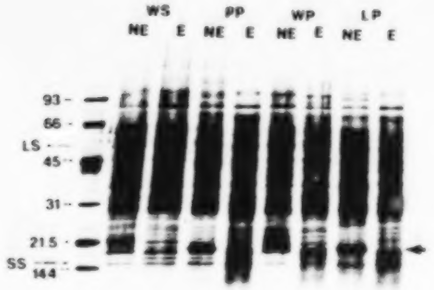

Figure 7. SDS-PAGE of soluble proteins extracted from white spruce (WS), pond pine (PP), white pine (WP), and loblolly pine (LP) embryogenic (E) and nonembryogenic (NE) calli.

In conclusion, it appears that proplastids or undifferentiated plastids are present in embryogenic conifer calli, while fully developed chloroplasts are found in green, non-embryogenic calli. Further work planned in our lab involves an attempt to identify the abundant protein found in non-embryogenic calli, along with other ongoing studies designed to look at differences in gene expression in embryogenic and non-embryogenic tissues. Aside from their use as markers of conifer somatic embryogenesis, both the chloroplast ultrastructure and soluble protein differences also offer a valuable opportunity to study the differentiation and development of both conifer embryos and chloroplasts.

Acknowledgments

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